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PRINCIPAL INVESTIGATOR: Ü [* ^ | Ã Ė Ą | ã] Ê Ú Ğ È

CONTRACTING ORGANIZATION: University of Ullah
P.O. Box 12345, Ullah, U.S.A.

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
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14. ABSTRACT Recombinant L-methioninase-annexin V and cytosine deaminase-annexin V fusion proteins have been produced in good purity and yield from E. coli. As indicated by measuring the dissociation constant (Kd), both purified FPs bind strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown in vitro. In vitro tests of both enzyme prodrug systems showed that significant killing of endothelial cells, MCF-7 cells, and MDA-MB-231 cells (~80% confluent) was obtained with very little or no effect of the prodrug when the FP was not present. The L-methioninase-annexin V/SeMet enzyme prodrug system, where SeMet is converted to methylselenol, was tested in vivo in SCID mice with implanted MDA-MB-231/GFP cancer cells using i.p. injection of the FP and the prodrug. The result was that tumor growth was arrested or greatly slowed during the 11-day treatment period, but tumor growth resumed at the end of treatment. These results indicate that the use of an enzyme prodrug system targeted to the tumor vasculature that yields a drug with a longer half-life than methylselenol may be more effective in treating breast tumors.					
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INTRODUCTION

The focus of this project is on the development of new ways to treat breast cancer with minimal or no side effects. There are two aims of the project: (1) Develop a novel enzyme prodrug and methionine-depletion combination cancer therapy in which the enzyme L-methioninase is targeted by the human protein annexin V to the breast tumor vasculature, using selenomethionine as the prodrug, and (2) develop a novel enzyme prodrug cancer therapy in which the enzyme cytosine deaminase is targeted by the human protein annexin V to the breast tumor vasculature, using 5-fluorocytosine as the prodrug. Annexin V is known to bind with high affinity to phosphatidylserine (PS) in phospholipids bilayers. PS has recently been shown to be expressed on the external surface of endothelial cells that line the blood vessels in tumors but is not expressed on the external surface of the vascular endothelium in normal organs. The enzyme L-methioninase catalyzes the conversion of methionine to methanethiol, α -ketobutyrate, and ammonia. It also catalyzes the conversion of selenomethionine (SeMet) to toxic methylselenol, α -ketobutyrate, and ammonia. Methylselenol has been shown to be cytotoxic to various cancer cells. Cytosine deaminase converts the prodrug 5-fluorocytosine to the cytotoxic drug 5-fluorouracil.

To accomplish the specific aims of this project, the L-methioninase-annexin V and cytosine deaminase-annexin V fusion proteins (FPs) was produced and purified, the strength of binding of each FP to human endothelial cells and two breast cancer cell lines *in vitro* was determined, the cytotoxicity *in vitro* of each FP in combination with its respective prodrug was determined for endothelial cells and two breast cancer cell lines, and the L-methioninase-annexin V/selenomethionine enzyme prodrug combination was tested in SCID mice with breast tumor xenografts for its effect as an enzyme prodrug by itself and also in combination with methionine-depletion therapy.

BODY

The research accomplishments for the project are summarized as follows:

Task 1 – Production of the recombinant L-methioninase-annexin V fusion protein to be tested

The production of purified recombinant L-methioninase-annexin V fusion protein is described in the annual reports for years 1 and 2 and in our publication (attached in Appendix I) about this research [1]. The yield of purified fusion protein was 30 mg from 1 liter of culture medium. The lyophilized fusion protein was determined to have good purity (>97%) and a methioninase specific activity of 1.0 U/mg of protein. The overall recovery yield of L-methioninase activity was found to be 50%.

Task 2 – Test of the function *in vitro* of annexin V in the L-methioninase-annexin V fusion protein

The strength of binding and stability of binding over 3 days of this FP for the three cell lines was reported in the annual report for year 2 and also in our publication about this research, which is attached in Appendix I [1]. The results for the dissociation constant K_d to measure the strength of binding indicate that the binding of the FP to these cells is relatively strong and that the binding to the endothelial cells is stronger than for either of the two breast cancer cell lines. The data for binding stability indicates that the binding of FP declined over 3 days for all three cell lines, with the MDA-MB-231 cancer cells showing the most rapid decline; however, the FP was still present at day 3 for all three cell lines.

Task 3 – Test of the anticancer activity *in vitro* of the L-methioninase-annexin V fusion protein in combination with selenomethionine prodrug on endothelial cells and breast tumor cells

Results of the ability of the enzyme prodrug system to eliminate human endothelial cells and breast cancer cells were given in the annual report for year 2 and also in our publication about this research, which is attached in Appendix I [1]. Significant killing of the cells (~80% confluent) was found at a SeMet concentration of 500 μ M for endothelial cells, 50 μ M for MCF-7 breast cancer cells, and 10 μ M for MDA-MB-231 breast cancer cells. With no FP present, significant cell killing was not observed for the endothelial cells and MDA-MB-231 cancer cells at up to 1000 μ M SeMet and for MCF-7 cancer cells at up to 500 μ M SeMet.

Task 4 – Test of the anticancer activity *in vivo* of the L-methioninase-annexin V fusion protein in combination with selenomethionine prodrug

Five tests of this enzyme prodrug therapy were performed in mice. The results of the first four of these tests were reported in the annual report for year 3. Before performing the fifth test, we did a test of growing MDA-MD-231/GFP breast tumors in three groups of female mice (three mice/group): Nu/J nude mice, nu/nu nude mice, and SCID mice. The tumors grew well in the SCID mice, and the tumors were fluorescent as indicated by an IVIS Spectrum small imaging system. The tumors did not grow in the Nu/J or nu/nu mice, and there was no fluorescence from these mice at the same time there was growth and fluorescence in the SCID mice. Therefore, the rest of the tests were performed using SCID mice.

The results of the fifth enzyme prodrug treatment, which used SCID mice on a normal diet, are shown in Figure 1. The tumors in mice receiving L-methioninase-annexin V and selenomethionine reduced in size throughout the treatment period, while mice receiving no treatment, L-methioninase-annexin V, or selenomethionine only steadily increased. One mouse in the selenomethionine group died during the treatment period. All mice in the treatment group tolerated the selenomethionine, presumably because the selenomethionine was converted to methylselenol and did not build up in tissue. Over the period of the study, all mice displayed a gain in weight. From 22 days after inoculation of the tumor cells until the end of the test, there

was a significant difference between the tumor volumes in the enzyme prodrug-treated mice compared to untreated mice ($p < 0.001$). During the 2-week observation period, mice in the treatment group showed tumor regrowth, indicating that not all the cancer cells were killed. Still, at the end of the study, tumors in the treated group were only a third of the size of the control groups. Histological analysis of three mice per control group and all seven treated mice revealed there were no metastatic lesions found in lungs or liver of any of the mice.

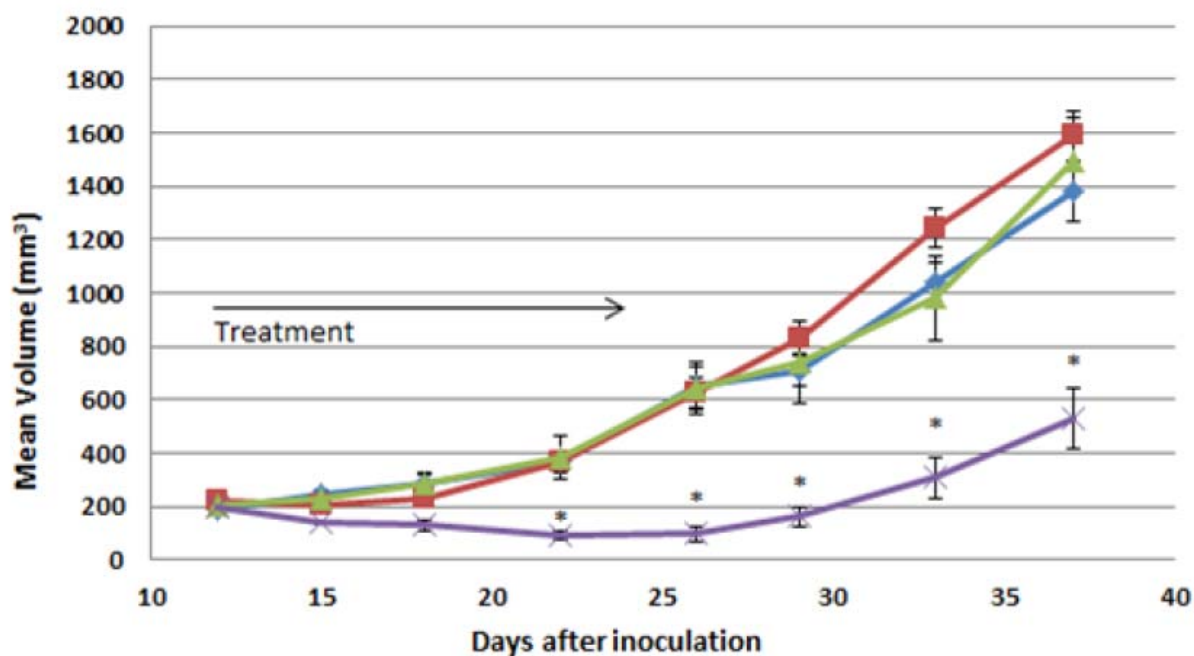


Figure 1. Enzyme prodrug treatment of MDA-MB-231 breast tumors in SCID mice. Treatment (denoted by the arrow) consisted of three consecutive cycles, where each cycle was as follows: 10 mg/kg L-methioninase-annexin V injected on day 0, and 10 mg/kg selenomethionine injected on days 1, 2, and 3. Untreated control (◇), L-methioninase-annexin V (□), selenomethionine (Δ), L-methioninase-annexin V + selenomethionine (×). Tumor volume data are presented as mean \pm SE ($n = 7$) with statistical significance compared to the control group denoted by * ($p < 0.001$).

A sixth test was performed of this enzyme prodrug therapy with reduced methionine available. The mice were placed on a diet deficient in methionine and choline supplemented with D,L-homocysteine 24 h prior to the start of treatment. The experimental design was similar to that above, with L-methioninase-annexin V fusion protein administered at 10 mg/kg, but the selenomethionine dosage was cut in half (5 mg/kg). Figure 2 shows the tumor volume curves obtained throughout the treatment and observation period. As expected, the L-methioninase-annexin V plus selenomethionine treatment resulted in a significant reduction in tumor growth compared to the untreated control. During the treatment period, the size of the tumors in the group of mice receiving the enzyme prodrug treatment increased slightly, and there was regrowth of the tumors after the end of the treatment. From 27 days after inoculation of the tumor cells until the end of the test for the untreated mice, there was a significant difference between the tumor volumes in the enzyme prodrug-treated mice compared to untreated mice ($p < 0.001$). At

the end of the test, the average size of the tumors in the group treated with L-methioninase-annexin V and selenomethionine was 42% of that in the control group, which is similar to what was found in the study with mice on a normal diet. In addition, treatment with only methioninase-annexin V did give a consistent reduction in tumor volume compared to untreated mice, but this reduction was small and not statistically significant. As in the study with mice on a normal diet, histological analysis showed no metastatic lesions in the lungs or liver in any mice, and all mice gained weight over the treatment period.

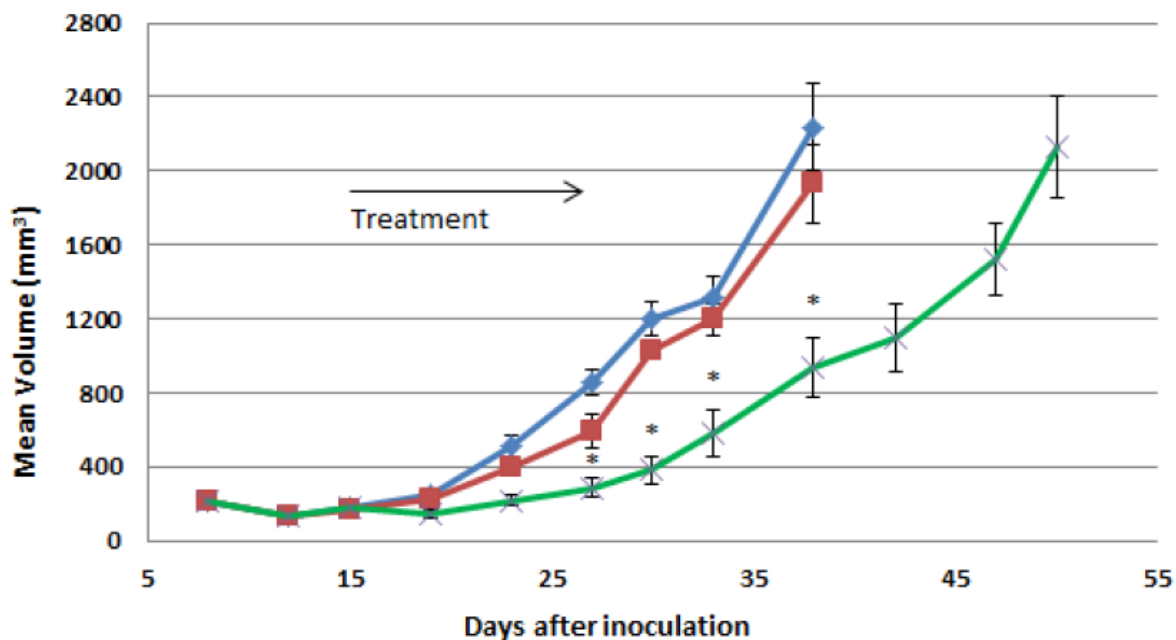


Figure 2. Enzyme prodrug treatment of MDA-MB-231 breast tumors in SCID mice on a diet deficient in methionine and choline supplemented with D,L-homocysteine. Treatment (denoted by the arrow) consisted of three consecutive cycles, where each cycle was as follows: 10 mg/kg L-methioninase-annexin V injected on day 0, and 5 mg/kg selenomethionine injected on days 1, 2, and 3. Untreated control (\diamond), L-methioninase-annexin V (\square), L-methioninase-annexin V + selenomethionine (\times). Tumor volume data are presented as mean \pm SE ($n = 7$) with statistical significance compared to the control group denoted by * ($p < 0.001$).

The procedure used for the detection of exposed phosphatidylserine in the tumor vasculature of mice is given in Appendix II. A stained section of an MDA-MB-231 tumor is shown in Figure 3. The presence of oxidized DAB, which is a dark brown color, is shown uniformly on the perimeter of a blood vessel within the tumor tissue, but there is none on the cancer cells surrounding the blood vessel. This indicates that biotinylated L-methioninase-annexin V is bound to the blood vessel, since the tissue slide was first treated with streptavidin-HRP. The bound HRP binds DAB as a substrate and then oxidizes it.

To validate that the enzyme prodrug is acting to cut off the flow of blood in the tumor vasculature, the blood flow through untreated and treated tumors in SCID mice was visualized by injecting a fluorescent dye i.p. and allowing the dye to circulate for 1 h before the imaging

(see the procedure in Appendix III). Images from untreated and treated mice with tumor xenografts, in which the DyLight 680 signal was spectrally unmixed using the IVIS Spectrum's Living Image software, are shown in Figure 4 (A, untreated; B, treated). The outline of the GFP signal showing the living tumor cell perimeter is in green on both figures. Untreated mice showed significant red fluorescence in the tumor area, indicating blood flow through the tumor vasculature, while treated mice showed much less red fluorescence in the tumor, indicating a lack of blood in the tumor region. The black hole on the left side of the mice in Fig. 4B is the tumor void of blood flow. The area of the untreated tumor was 93% red fluorescent, while that in the treated tumor was 33% fluorescent. Thus, based on the pictures showing red fluorescence, nearly all of the untreated tumor was receiving blood flow, compared to 33% receiving blood flow in the treated tumor.

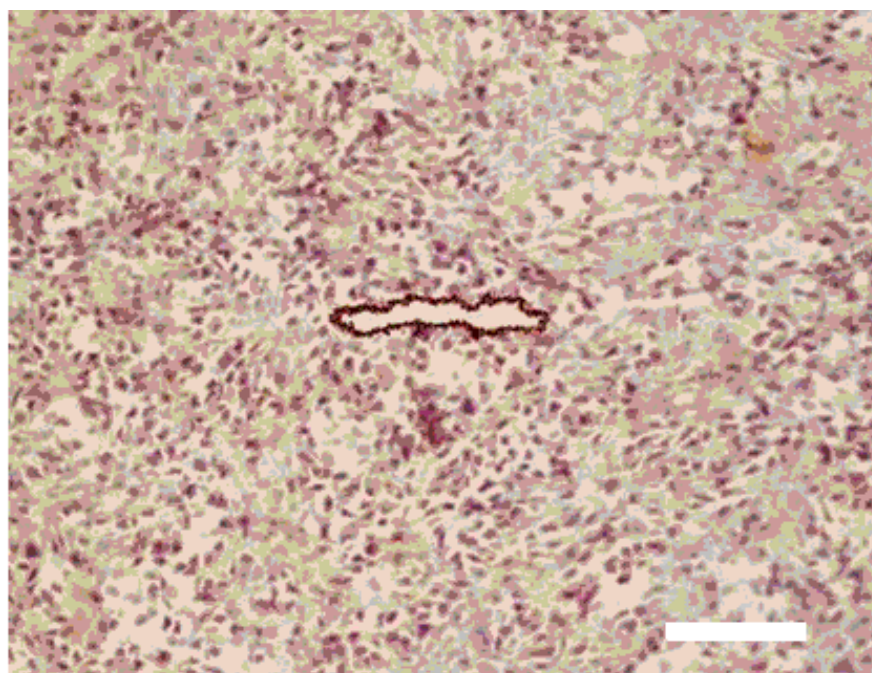


Figure 3. Detection of L-methioninase-annexin V bound to exposed PS on the surface of a blood vessel in an MDA-MB-231 breast tumor grown in SCID mice. Biotinylated fusion protein was injected i.p. Crysections were stained using streptavidin-HRP and developed with DAB, and counterstaining was done with hematoxylin. The periphery of the blood vessel is stained dark brown with DAB, indicating bound L-methioninase-annexin V. The scale bar represents 125 μm .

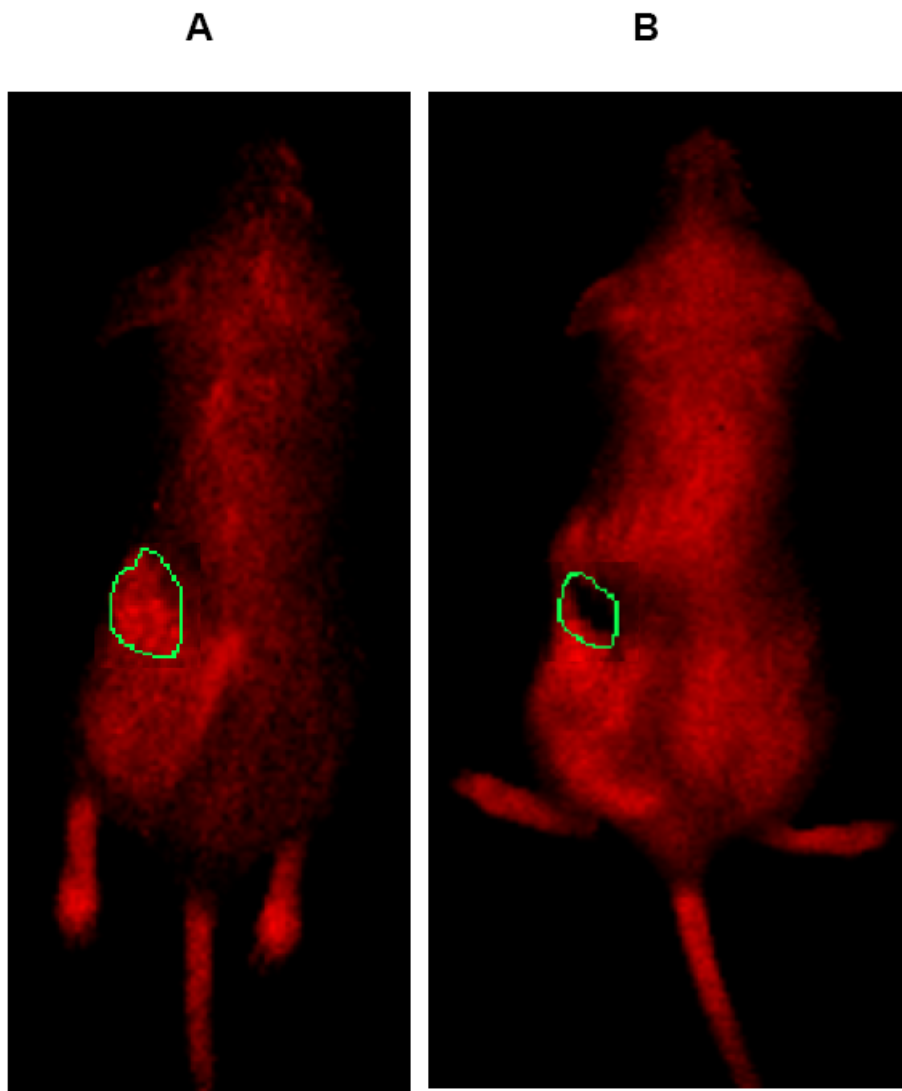


Figure 4. Blood flow through the tumor without (A) and with (B) enzyme prodrug treatment. Female SCID mice bearing MDA-MG-231 breast tumors were injected with DyLight 680 red fluorescent dye the day after the completion of the three-cycle enzyme prodrug treatment. The outline of the GFP signal showing the living tumor perimeter is shown in green. The area in red indicates the presence of blood flow.

The main finding to emerge from this study is that this enzyme prodrug therapy directed to the tumor vasculature was able to arrest or greatly slow the growth of MDA-MB-231 breast tumors during the 11-day treatment period, but tumor growth resumed at the end of treatment. The L-methioninase-annexin V fusion protein was designed to bind to the tumor vasculature, where the selenomethionine prodrug could be converted to a drug to attack the tumor vasculature and the tumor. Binding of L-methioninase-annexin V, labeled with biotin, to the tumor

vasculature after i.p injection was demonstrated by detection with streptavidin-HRP and DAB substrate. (Figure 3).

The results of the experiment to measure the blood flow in the untreated and treated tumor suggest that treated mice had compromised tumor vasculature resulting from the production of methylselenol from selenomethionine (Figure 4). This confirms a substantial cutoff of blood flowing through the treated tumor and means that a substantial degree of hypoxia was created in the treated tumor. Hypoxia in the tumor would result in a slowing of tumor growth or death of the tumor. Indeed, tumor growth was arrested or greatly slowed in both of the *in vivo* treatment tests (Figures 1 and 2). During hypoxic conditions, hypoxia-inducible factor-1 (HIF-1) induces gene expression in promoting tumor cell survival [2]. Several inhibitors of HIF-1 have been found, including digoxin. Therefore, this enzyme prodrug therapy potentially could be improved by using it in combination with an inhibitor of HIF-1.

The tumor growth observed after the end of enzyme prodrug treatment could be because the half-life of the methylselenol drug is so short that it was not be able to reach a significant percentage of cancer cells in the tumor by permeation and/or diffusion before being degraded. No data have been reported on the half-life of methylselenol, probably because it is highly reactive and therefore difficult to quantify and detect [3].

The tumor growth at the end of treatment observed in Figures 1 and 2 is consistent with findings for conventional antibody-directed enzyme prodrug therapy (ADEPT) systems, which typically inhibit tumor growth during treatment, but fail to kill all malignant cells, resulting in tumor regrowth. Vrudhula et al. [4], for example, used a monoclonal antibody to target β -lactamase in a lung adenocarcinoma model in athymic nude mice. A cephalosporin prodrug was used to generate toxic cephalosporin mustard, which led to tumors one-third the size of untreated tumors—very similar to what was obtained in the present study. A different type of mustard drug released by an antibody fragment-carboxypeptidase G2 conjugate was used in an enzyme prodrug therapy in athymic nude mice with colorectal tumor xenografts; tumor regression was obtained, but tumor regrowth was eventually observed [5]. Tumor regrowth could be a result of uneven distribution of the antibody-enzyme complex in the tumor. This type of distribution was shown in a study where a monoclonal antibody was injected i.v. in patients with malignant melanoma; the result was that the antibody was found to be heterogeneously distributed through the tumor, which was not primarily due to heterogeneity of antigen expression but could have been a result of differences in capillary wall permeability [6].

The test of combining a diet deficient in methionine and choline (supplemented with homocysteine) with this enzyme prodrug therapy did not result in an improvement compared to when mice were fed a normal diet (Figure 2), which is an indication that the injection of L-methioninase-annexin V was not frequent enough to give a consistently low concentration of methionine in the bloodstream. In a study by Kokkinakis et al. [7], growth of tumors in athymic nude mice could be arrested by administering L-methioninase twice daily over 12-h intervals. In the present study, L-methioninase-annexin V was administered on day 0, 4, and 8 during the treatment period.

The advantage of the enzyme prodrug system investigated here compared to conventional antibody-directed enzyme prodrug therapy (ADEPT) systems is that there is no requirement that a protein be transported out of the bloodstream and cross multiple barriers to reach the tumor; a fusion protein such as L-methioninase-annexin V will bind rapidly to exposed PS on endothelial cells in the tumor vasculature after being injected into the bloodstream. However, the full potential of enzyme prodrug therapy targeted to the tumor vasculature apparently has not been

realized in the present study because while there is evidence of a cutting off of the blood supply in the tumor using this therapy that leads to a slowing or halting of tumor growth, the methylselenol drug created by this therapy may not be reaching the tumor sufficiently to cause a reduction in tumor size. Therefore, an enzyme prodrug system that yields a drug with a longer half-life than methylselenol is warranted. One system that appears promising to test is purine nucleoside phosphorylase-annexin V/fludarabine, which results in 2-fluoroadenine as the anticancer drug. The half-life of 2-fluoroadenine consists of an alpha phase of 31 min and a beta phase of 114 min [8], which are sufficiently high for this drug to be transported to the tumor by diffusion and permeation from the tumor vascular wall where it is generated.

Another way this therapy could potentially be improved is by combining the enzyme prodrug treatment with docetaxel to increase the exposure of PS in the tumor vasculature and thus lead to a greater amount of the enzyme that is bound. Docetaxel has been found to about double the percentage of tumor vessels in mice that expose anionic phospholipids, while not leading to any exposure of these phospholipids in the vasculature of any of the normal organs [9]. Increasing the enzyme binding would lead to a greater concentration of the anticancer drug in the tumor and thus to greater tumor killing.

The results of the tests in mice shown in Figures 1-4 have been submitted for publication.

Task 5 - Production of the recombinant cytosine deaminase-annexin V fusion protein

The results of the production and purification of the cytosine deaminase-annexin V FP are described in our publication about this research, which is attached in Appendix IV [10]. The purity of the FP was estimated to be >96% using densitometry software analysis. The theoretical molecular weight of the FP monomer is 53 kDa, which is consistent with the SDS-PAGE result for the purified FP. The lyophilized FP was determined to have a cytosine deaminase specific activity of 18 U/mg of protein, which is consistent with the specific activity of purified cytosine deaminase that has been reported in the literature. The yield of cytosine deaminase was 50 mg/liter of starting cell culture, and the overall recovery yield of cytosine deaminase activity was >90%.

Task 6 - Test of the function *in vitro* of annexin V for the cytosine deaminase-annexin V fusion protein

The strength of binding and stability of binding over 3 days of this FP for the three cell lines are reported in our publication about this research, which is attached in Appendix IV [10]. The dissociation constant (K_d) for measuring the strength of binding for each cell line grown to ~80% confluence tested was obtained from the specific binding data to give the following results: 1.5 ± 0.2 nM, endothelial cells, 0.6 ± 0.4 nM for MCF-7 breast cancer cells, and 4.2 ± 1.8 nM for MDA-MB-231 breast cancer cells, indicating relatively strong binding between the FP and PS. In a control test, the specific binding assay was performed on endothelial cells that were 100% confluent, and the result was that the total and non-specific data were essentially identical, indicating no specific binding and therefore a lack of PS expression on the cell surface. The data for binding stability shows that the FP per cell declined over 3 days; however, binding was still measureable after 3 days.

Binding was also studied using the FP labeled with FITC (fluorescein isothiocyanate) [10]. A fluorescence image of MDA-MB-231 cells shows that the FP is bound to the surface of the cells and not internalized. By remaining on the cell surface and not being internalized, where it would be digested, the FP is able to convert 5-FC to 5-FU and thus lead to cytotoxicity of the cell and other cells surrounding it, by a bystander effect. Endothelial cells incubated with FITC-labeled FP were also observed to be fluorescent only at the cell surface when they were 70% confluent. No fluorescence was observed when endothelial cells were 100% confluent, indicating no binding of the FP and thus no expression of PS on the surface of the cells.

Task 7 - Test of the anticancer activity *in vitro* of the cytosine deaminase-annexin V fusion protein in combination with 5-fluorocytosine prodrug on endothelial cells and the two breast tumor cell lines

Results of the ability of the cytosine deaminase-annexin V/5-fluorocytosine enzyme prodrug system to eliminate human endothelial cells and breast cancer cells (~80% confluent) are presented in our publication about this research, which is attached in Appendix IV [10]. For the endothelial cells, significant killing was observed on day 9 for cells with FP plus 5-FC ($p < 0.001$), and killing was observed to be dose-dependent. The two breast cancer cell lines exhibited similar patterns of cell death. Both MCF-7 and MDA-MB-231 cells receiving the FP plus 5-FC showed significant cell killing that was dose-dependent, compared to the control group, on days 3, 6, and 9 ($p < 0.001$). By day 9, treatment with the FP plus 5-FC resulted in 4% and 12% cell viability at 1000 μM 5-FC for MCF-7 and MDA-MB-231 cancer cells, respectively. For all three cell lines, no significant cell death was found with 5-FC present and FP absent, or with 5-FC absent but FP present.

KEY RESEARCH ACCOMPLISHMENTS

- Purified recombinant L-methioninase-annexin V fusion protein (FP) was produced in good purity (>97%) and yield (30 mg/liter of starting culture broth).
- As indicated by measuring the dissociation constant (K_d), purified L-methioninase-annexin V binds strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown *in vitro*. Hydrogen peroxide was found to not be needed to induce exposure of PS on non-confluent endothelial cells. The following K_d values were determined: 0.5 nM for endothelial cells, 6.2 nM for MCF-7 cells, and 4.9 nM for MDA-MB-231 cells. The amount of L-methioninase-annexin V bound on the three cell lines *in vitro* was found to decline steadily over 3 days, but there was still some FP bound at day 3.
- In tests of the L-methioninase-annexin V/SeMet enzyme prodrug system *in vitro*, significant killing of the cells (~80% confluent) was found at a SeMet concentration of 500 μM for endothelial cells, 50 μM for MCF-7 breast cancer cells, and 10 μM for MDA-MB-231 breast cancer cells. With no FP present, significant cell killing was not observed for the endothelial cells and MDA-MB-231 cancer cells at up to 1000 μM SeMet and for MCF-7 cancer cells at up to 500 μM SeMet.

- A time profile of L-methioninase-annexin V in the bloodstream after i.p. injection in nude mice showed that the FP diminished to an undetectable level 8 hours after injection. The peak level of the FP was approximately 10 times greater for an injection of 10 mg/kg than for 1 mg/kg.
- In a test of the effectiveness of the L-methioninase-annexin V/SeMet enzyme prodrug system *in vivo* in SCID, the growth of implanted MDA-MB-231/GFP cancer cells was arrested or greatly slowed during the 11-day treatment period, but tumor growth resumed at the end of treatment. For mice on a normal diet, tumors in the treated group were only a third the size of those in the untreated group. The test of combining a diet deficient in methionine and choline (supplemented with homocysteine) with this enzyme prodrug therapy did not result in an improvement compared to when mice were fed a normal diet. Histological staining showed that L-methioninase-annexin V was bound on the tumor vasculature but not on the cancer cells surrounding this vasculature. A test of injecting a fluorescent dye at the end of the 11-day treatment showed a large reduction in blood flow in the tumor in the treated tumor compared to the untreated tumor.
- Purified recombinant cytosine deaminase V fusion protein was produced in good purity (>96%), yield (50 mg/liter of starting culture broth), and specific activity (18 U/mg of protein).
- Purified cytosine deaminase V binds strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown *in vitro* to ~80% confluence, as indicated by measuring the dissociation constant (K_d). The following K_d values were determined: 1.5 nM for endothelial cells, 0.6 nM for MCF-7 cells, and 4.2 nM for MDA-MB-231 cells. Using FP with FITC attached, binding to the surface of MDA-MB-231 cells was observed by fluorescence microscopy. Endothelial cells incubated with FITC-labeled FP were also observed to be fluorescent only at the cell surface when they were 70% confluent; no fluorescence was observed when endothelial cells were 100% confluent, indicating no binding of the FP and thus no expression of PS on the surface of the cells. This result for binding to endothelial cells indicates that dividing cells mimic exposure of PS in the tumor vasculature, while confluent cells mimic PS exposure in the normal vasculature. The binding of cytosine deaminase-annexin V to the three cell lines *in vitro* was found to decline steadily over 3 days, but there was still some FP bound at day 3.
- In tests of the cytosine deaminase-annexin V/5-FC enzyme prodrug system *in vitro* with ~80% confluent cells, significant killing of endothelial cells was observed on day 9 for cells with FP plus 5-FC. The two breast cancer cell lines exhibited similar patterns of cell death. Both MCF-7 and MDA-MB-231 cells receiving the FP plus 5-FC showed significant cell killing that was dose-dependent, compared to the control group, on days 3, 6, and 9 ($p < 0.001$). By day 9, treatment with the FP plus 5-FC resulted in 4% and 12% cell viability at 1000 μ M 5-FC for MCF-7 and MDA-MB-231 cancer cells, respectively. For all three cell lines, no significant cell death was found with 5-FC present and FP absent, or with 5-FC absent but FP present.

REPORTABLE OUTCOMES

Brent D. Van Rite, Yahya A. Lazrak, Magali L. Pagnon, Naveen R. Palwai, Peter S. McFetridge, and Roger G. Harrison, Enzyme Prodrug Therapy Designed to Target L-Methioninase to the Tumor Vasculature, *Cancer Letters*, 2011, 301, 177-184.

Brent D. Van Rite and Roger G. Harrison, Annexin V-targeted Enzyme Prodrug Therapy Using Cytosine Deaminase in Combination with 5-Fluorocytosine, 2011, *Cancer Letters*, 307, 53-61.

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CONCLUSION

Both recombinant L-methioninase-annexin V and cytosine deaminase-annexin V fusion proteins were shown to bind strongly to PS exposed on human endothelial cells and MCF-7 and MDA-MB-231 breast cancer cells (dissociation constants ranging from 0.5 to 6.2 nM). Using FITC-labeled cytosine deaminase-annexin V, binding on the surface of MDA-MB-231 breast cancer cells was observed by fluorescence microscopy. Human endothelial cells incubated with FITC-labeled FP were also observed to be fluorescent only at the cell surface when they were 70% confluent; no fluorescence was observed when endothelial cells were 100% confluent, indicating no binding of the FP and thus no expression of PS on the surface of the cells. This result for binding to endothelial cells indicates that dividing cells mimic exposure of PS in the tumor vasculature, while confluent cells mimic PS exposure in the normal vasculature. In a binding experiment over 3 days for each of the three cell lines, it was found that there was a steady decline in both FP's bound over this period, but there was still some FP bound at day 3. *In vitro* tests of both enzyme prodrug systems showed that significant killing of endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells (~80% confluent) was obtained with very little or no effect of the prodrug when the FP was not present.

The L-methioninase-annexin V/SeMet enzyme prodrug system was tested *in vivo* in SCID mice with implanted MDA-MB-231/GFP cancer cells using i.p. injection of the FP and the prodrug. The result was that tumor growth was arrested or greatly slowed during the 11-day treatment period but tumor growth resumed at the end of treatment. A similar result was obtained using mice on a methionine-deficient diet, indicating that the injection of L-methioninase-annexin V was not frequent enough to give a consistently low concentration of methionine in the bloodstream to cause cancer cell death by methionine depletion. The results showing L-methioninase-annexin V bound only on the tumor vasculature and showing a large reduction in blood flow in the tumor in the treated tumor compared to the untreated tumor indicate that this therapy is working as it was designed to do. However, the full potential of enzyme prodrug therapy targeted to the tumor vasculature apparently has not been realized in the present study because while there is evidence of a cutting off of the blood supply in the tumor using this therapy that leads to a slowing or halting of tumor growth, the methylselenol drug created by this therapy may not be reaching the tumor sufficiently to cause a reduction in tumor size. Therefore, an enzyme prodrug system that yields a drug with a longer half-life than methylselenol is warranted. One system that appears promising to test is purine nucleoside

phosphorylase-annexin V/fludarabine, which results in 2-fluoroadenine as the anticancer drug. The half-life of 2-fluoroadenine is in the 30-115 min range, which is sufficiently high for this drug to be transported to the tumor by diffusion and permeation from the tumor vascular wall where it is generated.

In conclusion, this new vascular-targeted enzyme prodrug therapy shows promise in being able to treat breast tumors, including those that have metastasized. The selection of a prodrug that is converted to a drug with a sufficient half-life to reach the cancer cells appears to be an important factor in being able to completely eradicate tumors.

PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Mohamad Cherry
 Roger Harrison
 Carla Kurkjian
 Peter McFetridge
 Vassilios Sikavitsas
 Brent Van Rite

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APPENDIX I

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Enzyme prodrug therapy designed to target L-methioninase to the tumor vasculature

Brent D. Van Rite, Yahya A. Lazrak, Magali L. Pagnon, Naveen R. Palwai, Luís F.F. Neves, Peter S. McFetridge, Roger G. Harrison*

Bioengineering Center and the School of Chemical, Biological and Materials Engineering, University of Oklahoma, Norman, OK, USA

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ABSTRACT

A new approach for enzyme prodrug therapy for cancer was tested using human endothelial cells and two breast cancer cell lines *in vitro*. The concept is to use the human annexin V protein to selectively target the enzyme L-methioninase to the tumor vasculature. The major finding was that enzyme prodrug treatment using the L-methioninase-annexin V fusion protein and selenomethionine as the prodrug over 3 days was shown to be lethal to the endothelial cells and the cancer cells, while having little or no effect with the prodrug but with no fusion protein present. Thus, this new approach appears promising.

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1. Introduction

Enzyme prodrug therapy for cancer was conceived as a way to avoid the systemic toxicity of chemotherapy. A type of enzyme prodrug therapy known as antibody-directed prodrug therapy (ADEPT) was first proposed in the 1980s as a means to confine the action of cytotoxic drugs to the tumor. In ADEPT, a tumor-associated antibody is linked to a drug-activating enzyme, and the resulting fusion protein is administered systemically and preferentially accumulates in the tumor [1]. A non-toxic prodrug is administered systemically and is converted in the tumor to a toxic drug by the enzyme. The enzyme should not have a human homolog to avoid prodrug activation in normal tissues. Although this system is appealing in principle, there have been clinical limitations of ADEPT, including

poor accessibility of the enzyme/antibody complex to the tumor [2].

To improve on the ADEPT concept for cancer treatment, we have developed a new approach that targets the enzyme prodrug therapy to the tumor vasculature. In this approach, human annexin V protein is fused to the enzyme moiety and used to bind to the anionic phospholipid phosphatidylserine (PS) on the surface of endothelial cells in the tumor vasculature. PS is typically exposed on the surface of the vasculature endothelium within blood vessels of tumors, but not on normal endothelium [3,4]. Also, it has been shown that annexin V, when injected systemically, localizes in the tumor vasculature and tumor cells [3,4]. For the enzyme component, we have chosen L-methionine gamma-lyase (accession #AAB03240), also known as L-methioninase, from *Pseudomonas putida*. This enzyme catalyzes the α,γ -elimination of L-methionine and selenomethionine and is not found in human tissue [5]. Methionine is converted to methanethiol, α -ketobutyrate, and ammonia, while selenomethionine is cleaved into toxic methylselenol, α -ketobutyrate, and ammonia [6]. Methylselenol has been shown to be approximately 200-fold more

* Corresponding author. Address: School of Chemical, Biological and Materials Engineering, University of Oklahoma, 100 E. Boyd, Room T335, Norman, OK 73019, USA. Tel.: +1 405 325 4367.

E-mail address: rharrison@ou.edu (R.G. Harrison).

cytotoxic to various human cancer cells than its prodrug [7] and is known to induce apoptosis in cancer cells [8,9].

Several mechanisms of action of this new system are envisioned. The methylselenol generated at the surface of the endothelial cells in the tumor leads to destruction of these cells, which leads to clotting in the tumor vasculature and a cutoff of the supply of oxygen to the tumor. Thus, the primary tumor and distant metastases can be treated simultaneously. Methylselenol is also carried to the tumor cells by fluid permeating through the artery wall because of the pressure gradient across the artery wall. The destruction of the endothelial cells releases tumor antigens directly in the bloodstream, which can cause the immune system to mount a systemic attack on any remaining tumor cells anywhere in the body; this immune response can be boosted by the administration of immunoadjuvants [10]. Finally, the L-methioninase will greatly reduce the supply of methionine in the tumor, which will weaken methionine-dependent cancer cells.

Using the methioninase-annexin V fusion protein as an enzyme prodrug system, here we report the characterization of this system by *in vitro* studies using human endothelial cells and two breast cancer cell lines that includes a determination of the dissociation constant (K_d) of binding to the cell surface, the stability of the binding, and an evaluation of the cytotoxicity of the enzyme prodrug system.

2. Materials and methods

2.1. Materials

Oligonucleotide primers were synthetically produced by the Molecular Biology Resource Facility at the Health Sciences Center of the University of Oklahoma. Linear pET-30 Ek/LIC vector, T4 DNA polymerase, HRV 3C protease, and NovaBlue and BL21(DE3) *E. coli* cells were obtained from Novagen (Madison, WI). BamHI restriction enzyme and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). HAAE-1 endothelial cells were from Coriell Cell Repositories (Camden, NJ). MCF-7 and MDA-MB-231 cells and cell culture media were obtained from the American Type Culture Collection (Manassas, VA). Streptavidin-HRP was purchased from KPL (Gaithersburg, MD). PCR and plasmid purification kits were from Qiagen (Vista, CA). Alamar Blue solution was obtained from BioSource (Camarillo, CA).

2.2. Construction of recombinant expression plasmid

The expression vector pET-30 Ek/LIC/METHANX, encoding the methioninase-annexin V fusion protein (FP), was constructed in the following manner: The DNA sequences encoding for the FP were amplified from pKK223-3/ATF-Meth [11] and pET-22b(+)/STFANX (obtained from Dr. Stuart Lind at the University of Colorado) by the polymerase chain reaction using the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN). The PCR primers used to create the fusion protein gene, connected by a flexible linker, were as follows:

(a) 5' primer for L-methioninase: 5'-GAC/GAC/GAC/AAG/ATG/CTT/GAA/GTC/CTC/TTT/CAG/GGA/CCC/CGC/GAC/TCC/CAT/AAC/AAC/ACC-3' (b) 3' primer for L-methioninase: 5'-GC/CGC/ATT/GGA/TCC/AGA/ACC/GCT/GCC/TGC/ACA/CGC/CTC/CAA/CGC/CAA/CTC/G-3' (c) 5' primer for annexin V: 5'-CG/ATT/CGC/GGA/TCC/GCA/CAG/GTT/CTC/AGA/GGC-3' (d) 3' primer for annexin V: 5'-GA/GGA/GAA/GCC/CGG/TTA/GTC/ATC/TTC/TCC/ACA/GAG/C-3'. The L-methioninase primers incorporated a 5' LIC cloning site (italics), an HRV 3C protease site (bold), and a 3' BamHI site (underlined). The annexin V primers added a 5' BamHI site (underlined) and a 3' LIC cloning site (italics).

The PCR products were purified using the QIAquick PCR purification kit, digested with BamHI restriction enzyme, and purified with the same kit. The pure, digested genes were ligated using T4 DNA ligase and run on an agarose gel. Using the QIAquick gel purification kit, the proper fragments were cut from the gel and purified. The pure methioninase-annexin V fusion gene was annealed to the pET-30 Ek/LIC linear vector using T4 DNA polymerase to create sticky ends and was transformed into competent NovaBlue cells. After successful transformation, plasmids containing the proper fusion gene insert were extracted from the NovaBlue cells using the QIAprep plasmid purification protocol and transformed into *E. coli* BL21(DE3) to be used as the host for protein expression. The final vector contains integrated thrombin and enterokinase cleavage sites, an N-terminal His-tag sequence for easy purification, and an engineered HRV 3C protease cleavage site that cleaves the sequence LEVLFQ|GP at the start of the methioninase-annexin V gene. The sequence of the FP gene was verified to be correct by DNA sequencing at the Oklahoma Medical Research Foundation (Oklahoma City, OK).

The expression vector pET-30 Ek/LIC/ANX that encodes annexin V was constructed using the same methods as for the pET-30 Ek/LIC/METHANX expression vector, as follows: The DNA sequence encoding for annexin V was amplified from pET-22b(+)/STFANX by the polymerase chain reaction, using two primers: (a) 5' primer: 5'-GAC/GAC/GAC/AAG/ATG/CTT/GAA/GTC/CTC/TTT/CAG/GGA/CCC/GCA/CAG/GTT/CTC/AGA/GGC-3' and (b) 3' primer: 5'-GA/GGA/GAA/GCC/CGG/TTA/GTC/ATC/TTC/TCC/ACA/GAG/C-3'.

2.3. Expression and purification of recombinant protein

Recombinant methioninase-annexin V FP and annexin V were produced and purified using the procedure of Zang [11]. This procedure uses immobilized metal affinity chromatography (IMAC) with immobilized Ni^{2+} to isolate the FP or annexin V. After cleavage by HRV 3C protease, purified FP or annexin V is collected in the flow-through of another IMAC. Any uncleaved FP or annexin V and HRV 3C protease, which also contains a His-tag, remained bound to the IMAC column.

2.4. Protein content and enzymatic activity determination

The Bradford assay from Bio-Rad (Hercules, CA) was used for all protein determinations throughout the purification using bovine serum albumin (BSA) as the standard. Samples were analyzed by denaturing gel electrophoresis

using the SDS–PAGE method with Coomassie blue staining [12]. The L-methioninase enzyme activity was measured using L-methionine as a substrate by the spectrophotometric determination of α -ketobutyrate with 3-methyl-2-benzothiazolone hydrazone hydrochloride [5].

2.5. Cell culture

Human HAAE-1 aortic endothelial cells were grown in F-12K medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS), 0.03 mg/ml endothelial cell growth supplement, and 0.1 mg/ml heparin. MCF-7 human breast cancer cells were maintained as monolayer cultures in Eagle's minimum essential medium containing Earle's balanced salt solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and supplemented with 10% FBS and 0.01 mg/ml bovine insulin. MDA-MB-231 human breast cancer cells were grown in Leibovitz's L-15 medium supplemented with 10% FBS and 2 mM L-glutamine. Penicillin (100 U/ml) and streptomycin (100 μ g/ml) were also added to each medium. HAAE-1 and MCF-7 cells were grown at 37 °C in a 5% CO₂ atmosphere, while MDA-MB-231 cells were grown without additional CO₂ at 37 °C, as recommended by the American Type Culture Collection.

2.6. Protein binding assay

Each cell line was grown to 70–80% confluence in T-75 flasks. Cells were transferred to 24-well culture plates (5×10^4 cells/well) and grown to 80–85% confluence. Cells were fixed to the plate using 0.25% glutaraldehyde in binding buffer (PBS with 2 mM Ca²⁺). Excess aldehyde groups were quenched using 50 mM NH₄Cl in binding buffer. Varying concentrations of biotinylated FP or annexin V, using SureLINK Chromophoric Biotin (KPL) in a 60 M excess during biotinylation, were diluted in binding buffer containing 0.5% BSA and incubated at 37 °C for 2 h. After washing with binding buffer with 0.5% BSA, streptavidin-HRP was added at 2 μ g/ml and incubated at room temperature for 1 h. Following washing with binding buffer, HRP was measured by adding the chromogenic substrate O-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.012 vol.%) in 0.05 mM phosphate-citrate buffer (pH 5.0). After 30 min at room temperature in the dark, the solution was transferred to a transparent 96-well plate, and the absorbance was read at 450 nm on a BioTek Synergy HT microtiter plate reader (Winooski, VT). All experiments had a blank that was subjected to the same procedure but with no FP or annexin V added. To determine non-specific binding, the same procedure was performed with no Ca²⁺ and 5 mM EDTA in the binding buffer, with the addition of a 1 h BSA (0.5%) pretreatment for the cancer cells prior to adding the FP or annexin V.

2.7. Binding stability assay

To assess how long the FP remains bound to the surface of the endothelial cells, a modified binding assay was used. Cells on 24-well plates were first incubated for 2 h at 37 °C

in a saturating concentration of biotinylated FP (100 nM) in complete growth medium with 2 mM Ca²⁺. The Alamar Blue assay (see *In vitro* enzyme prodrug cytotoxicity to cells) was done on separate sets of cells at days 0–3 to determine viability, followed by fixing with 0.25% glutaraldehyde in binding buffer. Excess aldehyde groups were quenched by incubation in a 50 mM NH₄Cl in binding buffer. The binding of FP was then quantified using streptavidin-HRP and OPD as above.

2.8. In vitro enzyme prodrug cytotoxicity to cells

The experiment was carried out over 3 days, using the same cells for each of the days. Cells were grown and plated in 24-well plates with respective growth media using the same procedure as for the FP binding assay (see above). Each medium was supplemented with 2 mM Ca²⁺ and 0.02 mM pyridoxal phosphate (since annexin V is Ca²⁺ dependent and pyridoxal phosphate is a cofactor for L-methioninase). On day 0, the cells were incubated in medium containing 100 nM FP for 2 h at 37 °C. The plates were washed and medium containing SeMet varying from 0 to 1000 μ M was added. The Alamar Blue assay was performed on all wells on day 1. The Alamar Blue assay was performed by adding Alamar Blue solution to each well to give 10% Alamar Blue and then incubated for 4 h at 37 °C. The solution was transferred to an opaque 96-well plate, and the fluorescence was read at 590 nm using excitation at 530 nm. The blank consisted of wells containing only medium and Alamar Blue solution. After the fluorescence reading, the plates were washed, replaced with fresh medium containing appropriate levels of SeMet, and placed in the incubator. The readings were taken every 24 h for the duration of the experiment.

2.9. Data analysis

All assays included wells in triplicate. To test differences in cell viability, a one-way ANOVA employing a Tukey–Kramer multiple comparisons test was performed using GraphPad InStat software (GraphPad; La Jolla, CA) with a significance level of $p < 0.001$.

3. Results

3.1. Protein expression and purification

An SDS–PAGE gel of the FP purification is shown in Fig. 1. Following expression of the fusion protein, the bacteria cells were harvested by centrifugation, resuspended in lysis buffer and sonicated. The resulting soluble protein fraction is shown in lane 1. The His-tagged fusion protein was bound to the Ni²⁺ column during the first chromatography step to allow some of the unwanted proteins to flow through into waste (lane 2), and then it was eluted (lane 3). After cleavage with the protease, the FP eluted during the flow-through (lane 4) of the second chromatography, while other proteins remained in the column. Dialysis of the flow-through, sterile filtration, endotoxin removal, and lyophilization ended the purification process. The yield of purified FP was 30 mg from 1 l of culture medium. The purity of the FP (lane 4) was estimated to be >97% using UN-SCAN-IT densitometry software analysis (Silk Scientific, Inc.). The theoretical molecular weight of the FP monomer is 80 kDa, which is consistent with the SDS–PAGE result for the purified FP (lane 4). The lyophilized FP was determined to have a methioninase specific activity of 1.0 U/mg of

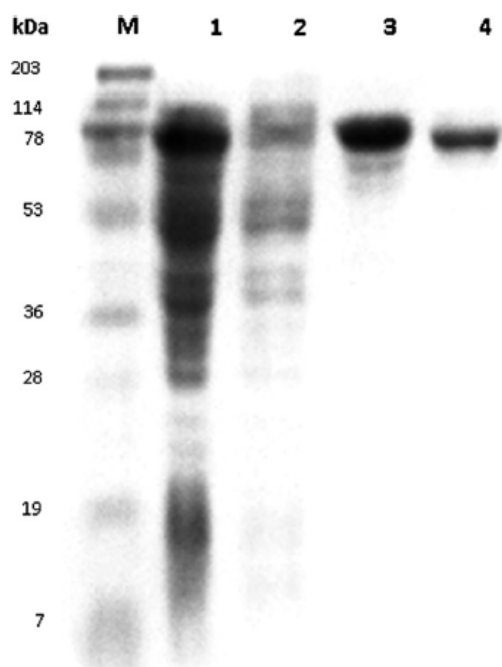


Fig. 1. SDS–PAGE gel of methioninase-annexin V purification. An 8% gel with Coomassie blue staining was used to determine approximate molecular mass of 10 μ l of purified samples. Lane 1, soluble proteins; lane 2, first chromatography flow-through; lane 3, first chromatography elution; lane 4, second chromatography flow-through; M marker proteins with molecular masses indicated in kiloDaltons (5 μ l).

protein. The overall recovery yield of L-methioninase activity was found to be 50%. L-methioninase activity remained relatively constant when stored in lyophilized form at -80°C .

Annexin V was expressed and purified using the same procedures as for the FP. The yield of purified annexin V was 80 mg per liter of culture medium, and the protein purity was estimated to be >97% using UN-SCAN-IT densitometry software analysis. The theoretical molecular weight of annexin V (35 kDa) is consistent with an SDS–PAGE analysis for purified annexin V (data not shown).

3.2. Specific binding and dissociation constant determination

The ability of the FP to bind to endothelial cells and breast cancer cells with PS exposed on the cell surface was evaluated by equilibrium binding experiments in which increasing concentrations of biotinylated FP were used. In initial experiments with endothelial cells, hydrogen peroxide was used at a low concentration (1 mM) to induce exposure of PS. In later experiments, the H_2O_2 was omitted with little change in the results; therefore, the data reported here is with no H_2O_2 added. No H_2O_2 was added in the experiments with the breast cancer lines, since it has been reported that cancer cells express PS *in vitro* [13,14].

A typical equilibrium binding result is shown in Fig. 2 for the binding of the FP to endothelial cells. The non-specific binding, obtained in the absence of Ca^{2+} , is subtracted from the total binding to obtain the specific binding. The dissociation constant (K_d) for each cell line tested was obtained from the specific binding data using GraphPad Prism 5 software to give the following results: 0.5 ± 0.2 nM for endothelial cells (with saturation $A_{450\text{ nm}} = 1.3$), 6.2 ± 1.6 nM for MCF-7 breast cancer cells (with saturation $A_{450\text{ nm}} = 0.9$), and 4.9 ± 0.9 nM (with saturation $A_{450\text{ nm}} = 1.8$) for MDA-MB-231 breast cancer cells. These results indicate that the binding of the FP to these cells is relatively strong. The binding of annexin V to endothelial cells was determined using the same procedures as for the FP. The K_d for the specific binding data was found using GraphPad Prism 5 software to be 0.9 ± 0.2 nM (with saturation $A_{450\text{ nm}} = 1.2$), which is sim-

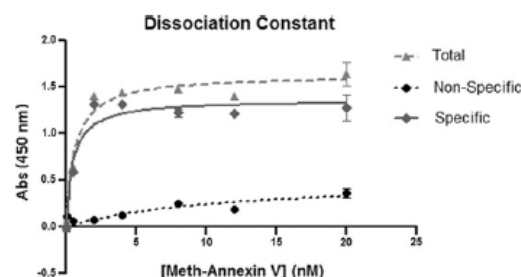


Fig. 2. Determination of FP binding strength to exposed PS on human endothelial cells. FP was biotinylated and streptavidin–HRP was used to quantify the binding. Total binding was obtained using 2 mM Ca^{2+} in the binding buffer. Non-specific binding was obtained by removing the Ca^{2+} from the binding buffer and replacing it with 5 mM of EDTA to chelate Ca^{2+} . Specific binding was obtained by subtracting the non-specific binding from the total binding. GraphPad Prism 5 software determined the specific binding to have a $K_d = 0.5 \pm 0.2$ nM. Data are presented as mean \pm SE ($n = 3$).

ilar to the K_d determined for the binding of the FP to endothelial cells. Literature values of annexin V binding alone to endothelial cells have been reported from 2.7–15.5 nM [15,16].

3.3. Fusion protein binding stability

The FP bound per cell was studied over 3 days for the three cell lines to determine the stability of binding by measuring the absorbance at 450 nm, determined by the binding assay, and dividing by the fluorescence at 590 nm, determined by the Alamar Blue assay (Fig. 3). The data in Fig. 3 indicate the binding of FP declined over 3 days for all three cell lines, with the MDA-MB-231 cancer cells showing the most rapid decline; however, the FP was still present at day 3 for all three cell lines. Cell viability, as measured by the Alamar Blue assay, was found to be linearly proportional to the number of cells (data not shown).

3.4. In vitro cytotoxicity of enzyme prodrug

The ability of the enzyme prodrug system to eliminate human endothelial cells and breast cancer cells was evaluated using a saturating concentration of fusion protein, followed by concentrations of SeMet ranging from 0 to 1000 μM (Figs. 4–6). The methionine concentration in the medium was set at a level (1000 μM) that would not lead to a significant decrease in cell viability because of methionine depletion with FP present.

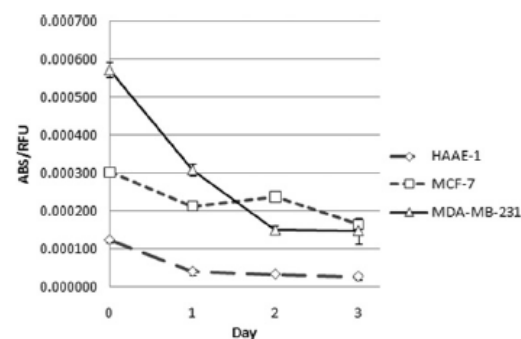


Fig. 3. Fusion protein binding stability. The Alamar Blue assay for cell viability was performed each day, followed by the binding assay to determine the duration of binding of annexin V to the exposed PS on the surface of each cell line. ABS/RFU is the absorbance at 450 nm, determined by the binding assay, divided by the relative fluorescence units at 590 nm, determined by the Alamar Blue assay. Data are presented as mean \pm SE ($n = 3$).

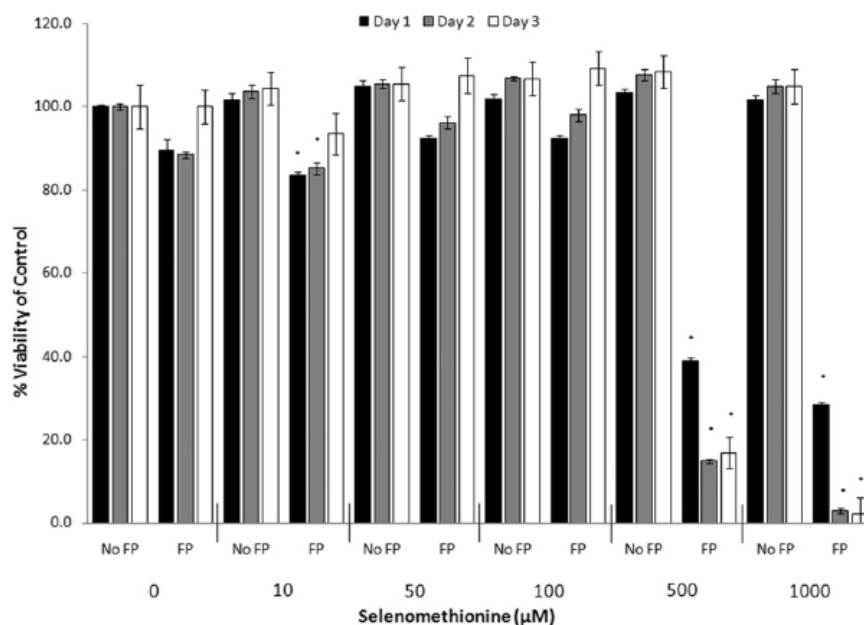


Fig. 4. Effect of SeMet conversion to methylselenol on HAAE-1 endothelial cells. Cells were grown in medium adjusted to 1000 μM of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 μM SeMet) on the same day, and statistical significance was denoted by # ($p < 0.001$). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean \pm SE ($n = 3$).

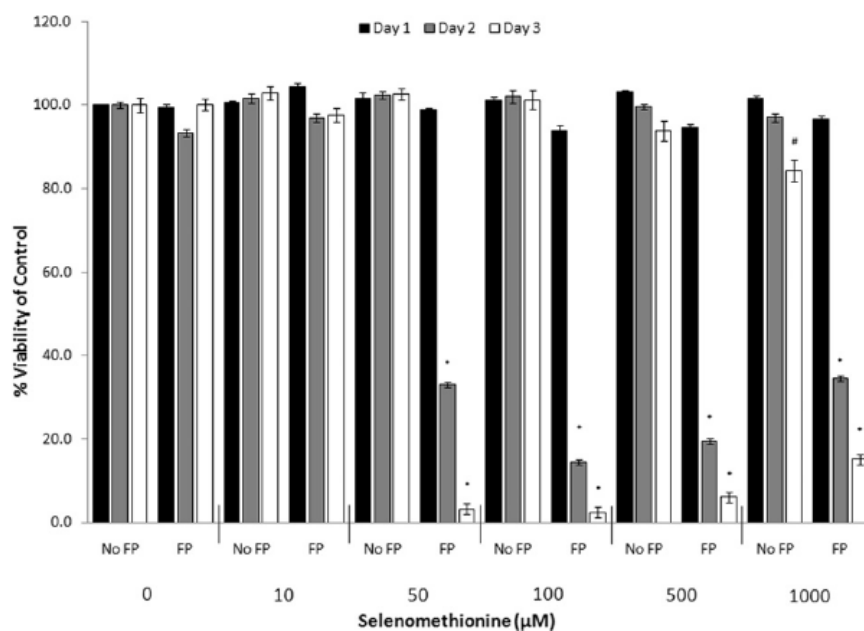


Fig. 5. Effect of SeMet conversion to methylselenol on MCF-7 breast cancer cells. Cells were grown in medium adjusted to 1000 μM of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 μM SeMet) on the same day, and statistical significance was denoted by # ($p < 0.001$). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean \pm SE ($n = 3$).

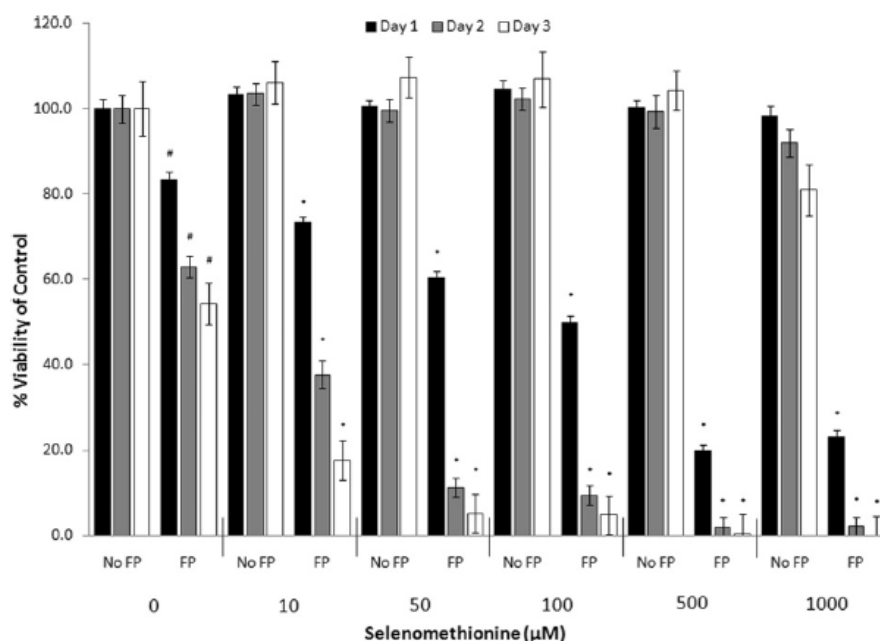


Fig. 6. Effect of SeMet conversion to methylselenol on MDA-MB-231 breast cancer cells. Cells were grown in medium adjusted to 1000 μ M of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 μ M SeMet) on the same day, and statistical significance was denoted by # ($p < 0.001$). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean \pm SE ($n = 3$).

Each of the cell lines metabolized the Alamar Blue to produce a fluorescence that was measured to quantify total cell viability. The fluorescence data from the Alamar Blue assay was expressed as a percentage of the fluorescence for the cells with no FP and 0 μ M SeMet (control). Cells that were treated with different SeMet concentrations but no FP were compared to the control on the same day, whereas cells that had the FP were compared to cells with the same SeMet concentration but no FP on the same day.

The cytotoxicity results for the endothelial cells are shown in Fig. 4. Treatment with the FP gave significant cell killing for 500 and 1000 μ M SeMet at days 1–3 ($p < 0.001$). With no FP present, significant cell cytotoxicity was not observed at the levels of SeMet tested.

Cytotoxicity results for the two breast cancer cell lines are shown in Figs. 5 and 6. For MCF-7 cells with FP present, there was significant killing at days 2 and 3 with 50–1000 μ M SeMet (Fig. 5, $p < 0.001$). Cell killing without FP present was not significant on day 3 until the SeMet concentration reached 1000 μ M. MDA-MB-231 cells showed a greater sensitivity to the SeMet than MCF-7 and endothelial cells (Fig. 6); significant cell cytotoxicity was observed with the FP present on days 1–3 with 10–1000 μ M SeMet ($p < 0.001$). Even without the addition of the SeMet, binding of the FP alone produced significant cell killing. With no FP present, cell killing did not occur until the SeMet level was 1000 μ M and was relatively small and not statistically significant ($p < 0.001$).

4. Discussion

In this study, we have shown that a novel FP developed for use in a new enzyme prodrug system binds relatively strongly to the surface of endothelial cells and two breast cancer cell lines. The K_d values for the two breast cancer cell lines are similar, while the K_d for the endothelial cells is about an order of magnitude lower, indicating much stronger binding. This low K_d value for the endothelial cells

is favorable for this enzyme prodrug system that is directed to the tumor vasculature.

The fact that the FP exhibited the same degree of binding to endothelial cells with or without hydrogen peroxide leads us to believe that endothelial cells grown *in vitro* by our techniques expose PS on their surface. It has been shown previously in tumor-bearing mice that PS is exposed on the external surface of vascular endothelial cells in tumor blood vessels but not on endothelial cells outside of the tumor; this was demonstrated using biotinylated annexin V [3,4]. We have found that the strength and specificity of binding of annexin V by itself (i.e. not as part of a fusion protein) to endothelial cells is very similar to that of the FP using the same procedures; and, therefore, there is no reason to believe that the FP will bind with any different strength or specificity *in vivo* compared to annexin V.

This binding stability data (Fig. 3) indicate that the FP was still bound to the cells after 3 days. The effectiveness of the enzyme in the FP during the test period is reinforced by the cytotoxicity data for the endothelial cells and two breast cancer cell lines (Figs. 4–6) for the SeMet concentrations at which there was a statistically significant decline in cell viability compared to the same SeMet concentrations with no FP present on the same day (indicated by a *); for this data there was in almost every case a decline in cell viability from days 1 to 2 and from days 2 to 3. The medium for the cells was changed at days 1 and 2, which means that the enzyme would have to produce additional methylselenol for more cells to be killed; if additional methylselenol were not produced, then no

methylselenol would be present (since the medium was changed), and additional cell killing would likely not occur.

In the enzyme prodrug test of cytotoxicity for the endothelial cells, which are the primary target in the tumor for this enzyme prodrug therapy, the cytotoxic effect was evident after only one day of treatment at 500 μ M SeMet. At either 500 μ M or 1000 μ M SeMet, there was no effect on endothelial cell viability with no FP present; this indicates that endothelial cells in the normal vasculature, which will not bind to the FP (since PS is not externalized), will not be affected by these concentrations of SeMet. At SeMet concentrations well below 500 μ M, however, the cancer cells will be killed by toxic methylselenol being carried across the artery wall by fluid permeation; the effect of methylselenol on normal cells outside of the tumor is expected to be minimal or none because it will be greatly diluted by the bloodstream before it reaches the normal cells. MDA-MB-231 breast cancer cells were found to be the more sensitive to the enzyme prodrug treatment than MCF-7 breast cancer cells, even producing killing when the FP was bound but no SeMet prodrug was added.

We have not found evidence that the sensitivity of the three cell lines to the enzyme prodrug treatment is dependent on the degree of exposure of PS on the cell surface. The saturation $A_{450\text{ nm}}$ values measured in the FP binding assay, which are a measure of the amount of FP bound to PS, do not correlate with the sensitivity to the enzyme prodrug treatment indicated in Figs. 4–6. Therefore, other cell properties besides the degree of PS exposure are contributing to the sensitivity to the effect of methylselenol.

Based on previous research, it is not expected that this enzyme prodrug therapy on the tumor vasculature will lead to toxicity to normal cells or organs. In a previous study using a monoclonal antibody to target anionic phospholipids in mice with implanted tumors, the growth of MDA-MB-231 and MDA-MB-435 breast tumors was inhibited, with no toxicity to the mice [17]. The blood vessels in MDA-MB-435 tumors implanted in mice have been shown to expose 35% of the anionic phospholipids [18]. PS is present at $>10^6$ molecules per cell [3], so even at 35% exposure, the number of anionic phospholipids on the surface of the cell is still very large.

Insight of how this enzyme prodrug system might be used therapeutically can be gained from the results of a previous study of treating mice with a methioninase cancer gene therapy with selenomethionine prodrug [7]. In this study, mice with implanted hepatoma tumor cells were treated with a recombinant adenovirus containing the *l*-methioninase gene and then were injected with SeMet once a day for 11 consecutive days, which led to inhibition of tumor growth and a significant prolongation of survival compared to mice not treated with SeMet. This dosing regimen is consistent with a previous finding that the selenium in human plasma peaks at 11 h on average after injection of sodium selenite [19]. No data have been reported on the half-life of methylselenol, probably because it is so highly reactive and therefore difficult to quantify and detect [20].

Several advantages of this new enzyme prodrug are envisioned. First, the FP can be easily administered through i.v. injection, and it will bind rapidly to its PS receptor in

contact with the bloodstream. Second, there would be minimal side effects since the drug is produced locally in the tumor and the prodrug concentration would be at a level that would not affect cells in other tissues besides the tumor. Third, it allows rapid targeting of metastatic tumors anywhere in the body. Fourth, it combines enzyme prodrug therapy with methionine-depletion therapy. Finally, the killing of endothelial cells in the tumor vasculature will cause the release of tumor antigen directly into the bloodstream, potentially leading to a response by the immune system against cancer cells throughout the body, which can be boosted by the administration of immunoadjuvants [10]. One disadvantage, common to all enzyme prodrug systems, is that the enzyme is not a human protein and thus may cause an immune response. This can be overcome by conjugating the *l*-methioninase part of the FP to polyethylene glycol (PEG). *l*-methioninase has, in fact, been conjugated to PEG, and the administration of PEG-*l*-methioninase to monkeys has been shown to eliminate anaphylactic reactions [21].

In conclusion, based on the data presented there is evidence that this therapy will be able to cause cytotoxicity to tumors wherever they occur in the body with minimal side effects. This new enzyme prodrug therapy system to treat cancer appears promising.

Conflicts of interest

None declared.

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APPENDIX II

Procedure for Detection of Externally Positioned Phosphatidylserine in MDA-MB-231 Tumor Vasculature

Female SCID mice (6 weeks old) were injected with 8×10^6 MDA-MB-231/GFP breast cancer cells subcutaneously into the left flank with an equal volume of matrigel, and the tumors were allowed to develop. Biotinylated L-methioninase-annexin V was injected i.p. at 10 mg/kg. One h after injection, the mice were placed under anesthesia, and the chest cavity was opened to expose the heart. A 27G butterfly needle was inserted into the left ventricle, and the ascending vena cava was cut. Heparinized saline with 2 mM Ca^{2+} was used to perfuse the circulation, followed by fixation with 0.25% glutaraldehyde in saline with calcium. The tumor was immediately resected and soaked in 20% sucrose and calcium overnight at 4°C, and the tumor samples were cryoembedded and cryosectioned using 8 μm slices. Slides were incubated with streptavidin-HRP to bind biotinylated L-methioninase-annexin V, washed, and developed with 3,3'-diaminobenzidine (DAB). Counterstaining was done with hematoxylin to visualize cell nuclei, and slides were preserved with ImmunoHistoMount (Santa Cruz Biotechnology, Santa Cruz, CA).

APPENDIX III

Procedure for Determination of Blood Flow through MDA-MB-231/GFP Tumors

SCID mice (n = 3 for treated and control groups) bearing MDA-MB-231/GFP tumors were subjected to the above treatment (10 mg/kg of L-methioninase-annexin V + 10 mg/kg of selenomethionine for three cycles). The day after the conclusion of the treatment, all six mice were injected i.p. with DyLight 680 fluorescent dye at 1 mg/kg. The dye was allowed to circulate for 1 h, after which time the amount of dye flowing through the control and treated tumors was documented using an IVIS Spectrum small animal imaging system.

APPENDIX IV

Brent D. Van Rite and Roger G. Harrison, Annexin V-targeted Enzyme Prodrug Therapy Using Cytosine Deaminase in Combination with 5-Fluorocytosine, 2011, Cancer Letters, 307, 53-61.



Annexin V-targeted enzyme prodrug therapy using cytosine deaminase in combination with 5-fluorocytosine

Brent D. Van Rite, Roger G. Harrison*

Bioengineering Center and the School of Chemical, Biological and Materials Engineering, University of Oklahoma, Norman, OK, USA

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ABSTRACT

A fusion protein, consisting of cytosine deaminase (CD) linked to human annexin V, was created for use in an enzyme prodrug therapy targeted to the tumor vasculature and associated cancer cells in the primary tumor and distant metastases. The major finding of this study is that the CD-annexin V fusion protein in combination with the prodrug 5-fluorocytosine has significant cytotoxic activity against endothelial cells and two breast cancer cell lines *in vitro* that expose phosphatidylserine on their surface. The cytotoxicity experiments verified this novel enzyme prodrug system has the ability to produce therapeutic levels of 5-fluorouracil and thus appears promising.

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1. Introduction

The anti-metabolite 5-fluorouracil (5-FU) is widely used in the treatment of solid tumors, including those of the breast, gastrointestinal system, head and neck, and ovary [1]. It has been established that 5-FU is converted inside the cell to the active metabolites fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP) [2]. These metabolites misincorporate into RNA and DNA and inhibit the nucleotide synthesis enzyme thymidylate synthase. Unlike using a drug that rapidly disrupts the membrane, 5-FU requires DNA and RNA replication to induce toxicity.

Cytotoxicity to non-cancerous tissues limits the dosage and frequency of drug administration. Common side effects of 5-FU are bone marrow suppression leading to neutropenia and infections, and gastrointestinal toxicities such as stomatitis, nausea, vomiting, and diarrhea [3]. To

circumvent problems related to systemic administration, new delivery strategies have been studied to produce 5-FU at the site of the tumor using an inert prodrug. As part of an enzyme prodrug system, 5-fluorocytosine (5-FC), a common antimicrobial compound known to exhibit high bioavailability [4], has been used in combination with cytosine deaminase (CD) to produce biologically relevant levels of 5-FU locally. CD has been used in antibody-directed enzyme prodrug therapy (ADEPT) by conjugating it to a single chain fragment variable (scFv) antibody [5,6]. In ADEPT, however, the antibody-enzyme complex must diffuse across the vascular wall to reach the tumor cells. In a study of the local distribution of a monoclonal antibody injected *i.v.* in patients with malignant melanoma, the antibody was found to be heterogeneously distributed through the tumor, which was not primarily due to heterogeneity of antigen expression but could have been a result of differences in capillary wall permeability [7].

Other studies have used CD-fusions for suicide gene-directed enzyme prodrug therapy (GDEPT) [8,9] and viral-directed enzyme prodrug therapy (VDEPT) [10,11]. Gene delivery is limited, however, by an inability to transfect large number of cells efficiently, while viral vectors have the potential of inducing severe immune responses and

* Corresponding author. Address: School of Chemical, Biological and Materials Engineering, University of Oklahoma, 100 E. Boyd, Room T335, Norman, OK 73019, USA. Tel.: +1 405 325 4367.

E-mail address: rharrison@ou.edu (R.G. Harrison).

also suffer from a limited amount and size of plasmid DNA [12].

As a potential new enzyme prodrug system designed to have cytotoxic effects on the primary breast tumor and distant metastases with minimal side effects, we linked yeast CD to human annexin V protein, known to bind specifically to the anionic phospholipid phosphatidylserine (PS), to create a fusion protein (FP). Yeast CD was chosen since previous work has demonstrated a greater ability of yeast CD to convert 5-FC to 5-FU than bacterial CD [13]. PS is exposed on the surface of vascular endothelial cells within the blood vessels of tumors, but not on normal endothelium [14,15]. There is also abundant evidence that PS is expressed on the outer surface of tumor cells [16,17]. Intravenous injection of the FP will allow annexin V to bind rapidly to exposed PS on endothelial cells in the tumor vasculature, and the FP will also be transported from the bloodstream to tumor cells via the well known enhanced permeability and retention (EPR) effect [18]. Following clearance of unbound FP, 5-FC would be injected and converted to 5-FU within the tumor both at the surface of the tumor vasculature and at the surface of the cancer cells.

In the present study, human endothelial cells and MCF-7 and MDA-MB-231 breast tumor cells were used to characterize binding and stability of binding of this novel yeast CD-annexin V fusion protein. FP binding data (K_D) suggests relatively strong binding to exposed PS, and the FP stays bound at least 3 days. The addition of 5-FC prodrug following FP binding to PS demonstrated the ability to kill the three cell lines by generating toxic 5-FU.

2. Materials and methods

2.1. Materials

Oligonucleotide primers were synthetically produced by Integrated DNA Technologies (Coralville, IA). Linear pET-30 Ek/LIC vector, T4 DNA polymerase, HRV 3C protease, and NovaBlue and BL21(DE3) *Escherichia coli* cells were obtained from EMD Chemicals (Gibbstown, NJ). BamHI restriction enzyme and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). HAAE-1 endothelial cells were from Coriell Cell Repositories (Camden, NJ). MCF-7 and MDA-MB-231 cells and cell culture media were obtained from the American Type Culture Collection (Manassas, VA). Streptavidin-HRP was purchased from KPL (Gaithersburg, MD). PCR and plasmid purification kits were from Qiagen (Vista, CA). Alamar Blue solution was obtained from BioSource (Camarillo, CA). 5-FC and 5FU were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Construction of recombinant expression plasmid

The expression vector pET-30 Ek/LIC/yCD-ANX, encoding the cytosine deaminase-annexin V fusion protein (FP), was constructed in the following manner: The DNA sequences encoding for the FP were amplified from pQE30Xa/yCD [19] (obtained from Dr. Maurizio Cianfriglia at the Superior Health Institute, Rome, Italy) and pET-22b(+)/STFANX (obtained from Dr. Stuart Lind at the

University of Colorado) by the polymerase chain reaction using the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN). The following PCR primers were used to create the fusion protein gene, connected by a flexible, six-amino acid linker: (a) 5' primer for cytosine deaminase: 5'-GAC/GAC/GAC/AAG/ATG/CIT/GAA/GTC/CTC/TTT/CAG/GGA/CCC/GTG/ACA/GGG/GGA/ATG/GCA/AGC-3' (b) 3' primer for cytosine deaminase: 5'-GC/CGC/ATT/GGA/TCC/AGA/ACC/GTC/GCC/CTC/ACC/AAT/ATC/TTC/AAA/CC-3' (c) 5' primer for annexin V: 5'-CG/ATT/CGC/GGA/TCC/GCA/CAG/GTT/CTC/AGA/GGC-3' (d) 3' primer for annexin V: 5'-GA/GGA/GAA/GCC/CGG/TTA/GTC/ATC/TTC/TCC/ACA/GAG/C-3'. The cytosine deaminase primers incorporated a 5' LIC cloning site (italics), an HRV 3C protease site (bold), and a 3' BamHI site (underlined). The annexin V primers added a 5' BamHI site (underlined) and a 3' LIC cloning site (italics).

Using the QIAquick PCR purification kit, PCR products were purified, digested with BamHI restriction enzyme, and purified with the same kit. Pure, digested genes were ligated using T4 DNA ligase and run on an agarose gel. The proper fused gene segment was cut from the gel and purified using the QIAquick gel purification kit. After annealing the pure cytosine deaminase-annexin V fusion gene to the pET-30 Ek/LIC linear vector using T4 DNA polymerase to create sticky ends, competent NovaBlue cells were transformed. Following successful transformation, plasmids containing the proper fusion gene insert were extracted from the NovaBlue cells using the QIAprep plasmid purification protocol. For host protein expression, *E. coli* BL21(DE3) cells were transformed with the plasmid. The final vector contains integrated thrombin and enterokinase cleavage sites, an N-terminal His-tag sequence for easy purification, and an engineered HRV 3C protease cleavage site for cleaving the sequence LEVLFQ↓GP at the start of the cytosine deaminase-annexin V gene. Sequencing by the Oklahoma Medical Research Foundation (Oklahoma City, OK) verified the gene sequence to be correct.

2.3. Expression and purification of recombinant protein

Recombinant cytosine deaminase-annexin V FP was produced and purified using the procedure of Zang et al. [20], using immobilized metal affinity chromatography (IMAC) with immobilized Ni^{2+} to isolate the FP. After cleavage by HRV 3C protease, purified FP is collected in the flow-through of a second IMAC. Uncleaved FP and the protease, which retain the His-tag, bind to the IMAC column and allow for collection of only cleaved FP.

2.4. Protein content and enzymatic activity determination

The BCA Protein assay from ThermoScientific (Waltham, MA) was used for all protein determinations throughout the purification using bovine serum albumin (BSA) as the standard. Samples were analyzed by denaturing gel electrophoresis using the SDS-PAGE method with Coomassie blue staining [21]. The cytosine deaminase enzyme activity was measured as described by Senter et al. [22]. Enzyme solution (225 μl) was added to PBS at 37 °C containing 0.5 mg/ml 5-FC for a total volume of 1 ml and

incubated at 37 °C for 30 min. A 50 μ l sample was quenched in 1 ml of 0.1 N HCl. From that, 250 μ l was removed, and its absorbance was measured at 255 and 290 nm using a BioTek Synergy HT microtiter plate reader (Winooski, VT). The concentration of 5-FU in quenched solutions was calculated from the formula given by Senter et al. One unit of enzyme activity is defined as 1 μ mol of 5-FU formed/min at 37 °C.

2.5. Cell culture

Human HAAE-1 aortic endothelial cells were grown in F-12 K medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS), 0.03 mg/ml endothelial cell growth supplement, and 0.1 mg/ml heparin. MCF-7 human breast cancer cells were maintained as monolayer cultures in Eagle's minimum essential medium containing Earle's balanced salt solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and supplemented with 10% FBS and 0.01 mg/ml bovine insulin. MDA-MB-231 human breast cancer cells were grown in Leibovitz's L-15 medium supplemented with 10% FBS and 2 mM L-glutamine. Penicillin (100 U/ml) and streptomycin (100 μ g/ml) were also added to each medium. HAAE-1 and MCF-7 cells were grown at 37 °C in a 5% CO₂ atmosphere, while MDA-MB-231 cells were grown without additional CO₂ at 37 °C, as recommended by the American Type Culture Collection.

2.6. Fusion protein binding assays

Each cell line was grown in T-75 flasks to 70–80% confluence, transferred to 24-well culture plates at 5×10^4 cells/well, and grown to 80–85% or 100% (control) confluence. Cells were fixed to the plate using 0.25% glutaraldehyde in binding buffer (PBS with 2 mM Ca²⁺), and unreacted aldehyde groups were quenched using binding buffer with 50 mM NH₄Cl. Prior to adding the FP, a 1 h pre-treatment of BSA (0.5%) in PBS was carried out at 37 °C. Varying concentrations (0–20 nM) of biotinylated FP, using SureLINK Chromophoric Biotin (KPL) for biotinylation, were diluted in binding buffer containing 0.5% BSA and incubated at 37 °C for 2 h. After washing with binding buffer with 0.5% BSA, streptavidin-HRP was added at 2 μ g/ml and incubated at room temperature for 1 h. Following a final wash with binding buffer, HRP was quantified by adding the chromogenic substrate O-phenylenediamine (0.4 mg/ml) in 0.05 mM phosphate-citrate buffer (pH 5.0) containing 0.012% hydrogen peroxide to initiate the reaction. After 30 min at room temperature in the dark, 100 μ l of the solution was transferred to a transparent 96-well plate, and the absorbance was read at 450 nm on a microtiter plate reader. To determine non-specific binding, the same procedure was performed with no Ca²⁺ and 5 mM EDTA in the binding buffer. All experiments had a blank that was subjected to the same procedure but with no FP added.

As further confirmation of FP binding to the cell membrane, MDA-MB-231 and endothelial cells were incubated for 2 h with FP tagged with FITC, following the protocol

provided with the FITC labeling kit from Pierce (Rockford, IL). A fluorescence microscope equipped with a Nikon DXM1200F digital camera was used for documentation of fluorescence.

2.7. Binding stability assay

A modified binding assay was used to determine the duration of FP binding to the surface of the three cell lines. Cells on 24-well plates were first incubated for 2 h at 37 °C in a saturating concentration of biotinylated FP (100 nM) in complete growth medium with 2 mM Ca²⁺. The Alamar Blue assay was done on separate sets of cells at day 0, 1, 2 and 3 to determine viability, followed by fixing with 0.25% glutaraldehyde in binding buffer. Unreacted aldehyde groups were quenched by incubation with 50 mM NH₄Cl in binding buffer. The binding of FP was then quantified using streptavidin-HRP and OPD as above. The Alamar Blue assay was performed by adding Alamar Blue solution to each well to give 10% Alamar Blue and then incubated for 4 h at 37 °C. A portion of the solution (250 μ l) was transferred to an opaque 96-well plate, and the fluorescence was read at 590 nm using excitation at 530 nm. The blank consisted of wells containing only medium and Alamar Blue solution.

2.8. In vitro enzyme prodrug cytotoxicity to cells

The experiment was carried out over 9 days using the same set of cells. Cells were grown and plated out in 24-well plates with respective growth media using the same procedure as for the FP binding assay above. Each medium was supplemented with 2 mM Ca²⁺ since annexin V is Ca²⁺ dependent. On day 0, the cells were incubated in medium containing 100 nM FP for 2 h at 37 °C. The plates were washed and medium containing 5-FC or 5-FU varying from 0 to 2000 μ M was added. Cells receiving 5-FU did not receive FP. Each day throughout the experiment, the medium was replaced with fresh medium containing 5-FC or 5-FU. The Alamar Blue assay was performed on all wells on days 3, 6, and 9 (as above). After the fluorescence readings on days 3 and 6, the plates were washed, and the cells were incubated with new FP using the same procedures as before. The cells were washed again, and fresh medium containing appropriate levels of 5-FC or 5-FU was added before further incubation.

2.9. Data analysis

All assays included wells in triplicate. To test differences in cell viability, a one-way ANOVA employing a Tukey-Kramer multiple comparisons test was performed using GraphPad InStat software (GraphPad; La Jolla, CA) with a significance level of $p < 0.001$.

3. Results

3.1. Protein expression and purification

A representative SDS-PAGE gel of the FP purification is shown in Fig. 1. Following expression of the fusion protein, the BL21 (DE3) cells were harvested by centrifugation, resuspended in lysis buffer and

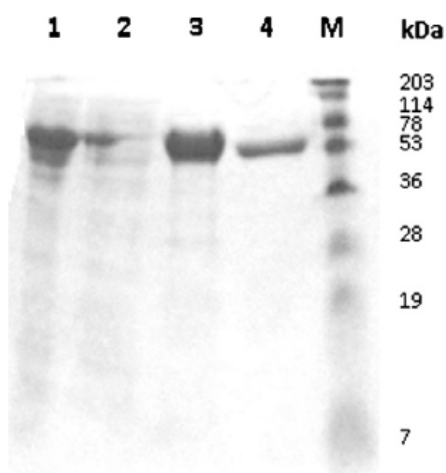


Fig. 1. SDS-PAGE gel of cytosine deaminase-annexin V purification. An 8% gel with Coomassie blue staining was used to determine approximate protein molecular masses in 10 μ l samples. Lane 1, soluble proteins; lane 2, first chromatography flow-through; lane 3, first chromatography elution; lane 4, second chromatography flow-through; M marker proteins with molecular masses indicated in kiloDaltons (5 μ l).

sonicated. The resulting soluble protein fraction is shown in lane 1. The His-tag attached to the fusion protein was bound to the Ni^{2+} column during the first chromatography step to allow some of the unwanted proteins to flow through into waste (lane 2), and then it was eluted (lane 3). After cleavage with the protease, the FP eluted during the flow-through (lane 4) of a second chromatography, while other proteins remained in the column. Dialysis of the flow-through, sterile filtration, and lyophilization ended the purification process. The yield of purified FP was 50 mg from 1 L of culture medium. The purity of the FP (lane 4) was estimated to be >96% using UN-SCAN-IT densitometry software analysis (Silk Scientific, Inc). The theoretical molecular weight of the FP monomer is 53 kDa, which is consistent with the SDS-PAGE result for the purified FP (lane 4). The lyophilized FP was determined to have a cytosine deaminase specific activity of 18 U/mg of protein. The overall recovery yield of cytosine deaminase activity was found to be >90%. Enzyme activity remained relatively constant when stored for 4 months in lyophilized form at -80°C .

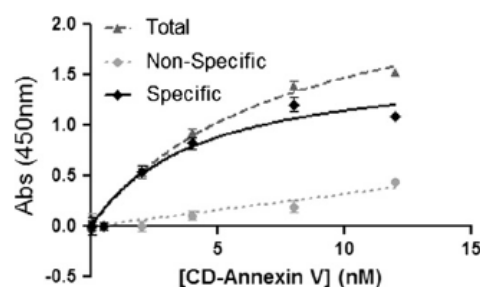


Fig. 2. Determination of FP binding strength to exposed PS on MDA-MB-231 breast cancer cells. FP was biotinylated, and streptavidin-HRP was used to quantify the binding. Total binding was obtained using 2 mM Ca^{2+} in the binding buffer. Non-specific binding was obtained by removing the Ca^{2+} from the binding buffer and replacing it with 5 mM of EDTA to chelate Ca^{2+} . Specific binding was obtained by subtracting the non-specific binding from the total binding. GraphPad Prism 5 software determined the specific binding to have a $K_d = 4.2 \pm 1.8$ nM (SE). Data are presented as mean \pm SE ($n = 3$).

3.2. Specific binding and dissociation constant determination

The ability of the FP to bind to exposed PS on endothelial cells and breast cancer cells *in vitro* was determined by equilibrium binding experiments using increasing concentrations of biotinylated FP. We previously determined that endothelial cells cultured *in vitro* with our methods expose PS [23]; thus hydrogen peroxide was not used to induce exposure of PS. It has been reported that cancer cells express PS *in vitro* [16,17].

A sample equilibrium binding result is shown in Fig. 2 for MDA-MB-231 breast cancer cells. The non-specific binding, obtained in the absence of Ca^{2+} and presence of EDTA, is subtracted from the total binding to obtain the specific binding. The dissociation constant (K_D) for each cell line tested was obtained from the specific binding data by GraphPad Prism 5 software to give the following results: 1.5 ± 0.2 nM (with saturation $A_{450\text{nm}} = 0.6$) for endothelial cells, 0.6 ± 0.4 nM (with saturation $A_{450\text{nm}} = 0.5$) for MCF-7 breast cancer cells, and 4.2 ± 1.8 nM (with saturation $A_{450\text{nm}} = 1.2$) for MDA-MB-231 breast cancer cells, indicating relatively strong binding between the FP and PS. In a control test, the specific binding assay was performed on endothelial cells that were 100% confluent. The result was that the total and non-specific data were essentially identical, indicating no specific binding and therefore a lack of PS expression on the cell surface (data not shown).

To illustrate binding of the FP, MDA-MB-231 cells were incubated with FITC-labeled FP. Fig. 3 shows a light microscopy image (left) of MDA-MB-231 cells and a fluorescence microscopy image (right) of FP bound to PS on the cell surface. Endothelial cells incubated with

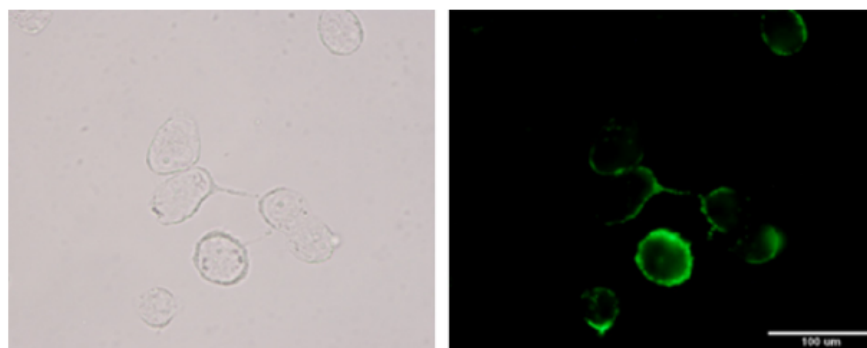


Fig. 3. Visualization of fluorescent FP binding to MDA-MB-231 breast cancer cells. Cells were seeded onto a cover glass and allowed to adhere. FP labeled with FITC was added, allowed to bind for 2 h, and washed to remove unbound FP. Corresponding light (left) and fluorescence (right) images were acquired with a 40 \times objective to verify FP binding to the cell surface. The bar represents 100 μ m.

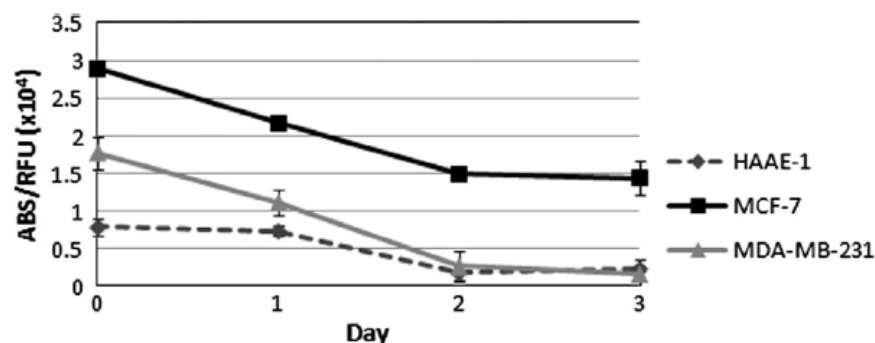


Fig. 4. Fusion protein binding stability. The Alamar Blue assay for cell viability was performed each day, followed by the binding assay to determine the duration of binding of the FP to exposed PS on the surface of each cell line. ABS/RFU is the absorbance at 450 nm, determined by the binding assay, divided by the relative fluorescence units at 590 nm, determined by the Alamar Blue assay. Data are presented as mean \pm SE ($n = 3$).

FITC-labeled FP were also observed to be fluorescent only at the cell surface when they were 70% confluent (data not shown). No fluorescence was observed when endothelial cells were 100% confluent, indicating no binding of the FP and thus no expression of PS on the surface of the cells.

3.3. Fusion protein binding stability

The FP bound per cell was studied over 3 days for the three cell lines to determine the stability of binding. The binding assay data (absorbance at 450 nm) was divided by the Alamar Blue fluorescence data for cell viability (relative fluorescence at 590 nm) (Fig. 4). The endothelial cells and

MCF-7 cancer cells had the lowest and highest amount of initial binding, respectively. For all three cell lines, the binding of the FP per cell declined over 3 days; however, binding was still measurable after 3 days. The number of viable cells and fluorescence signal produced by the Alamar Blue assay were found to exhibit a linear relationship (data not shown).

3.4. In vitro cytotoxicity of enzyme prodrug

Cell viability was determined on days 3, 6, and 9 to demonstrate the cytotoxic effects of 5-FC, with or without the fusion protein, against human endothelial cells and breast cancer cells (Figs. 5–7). In addition,

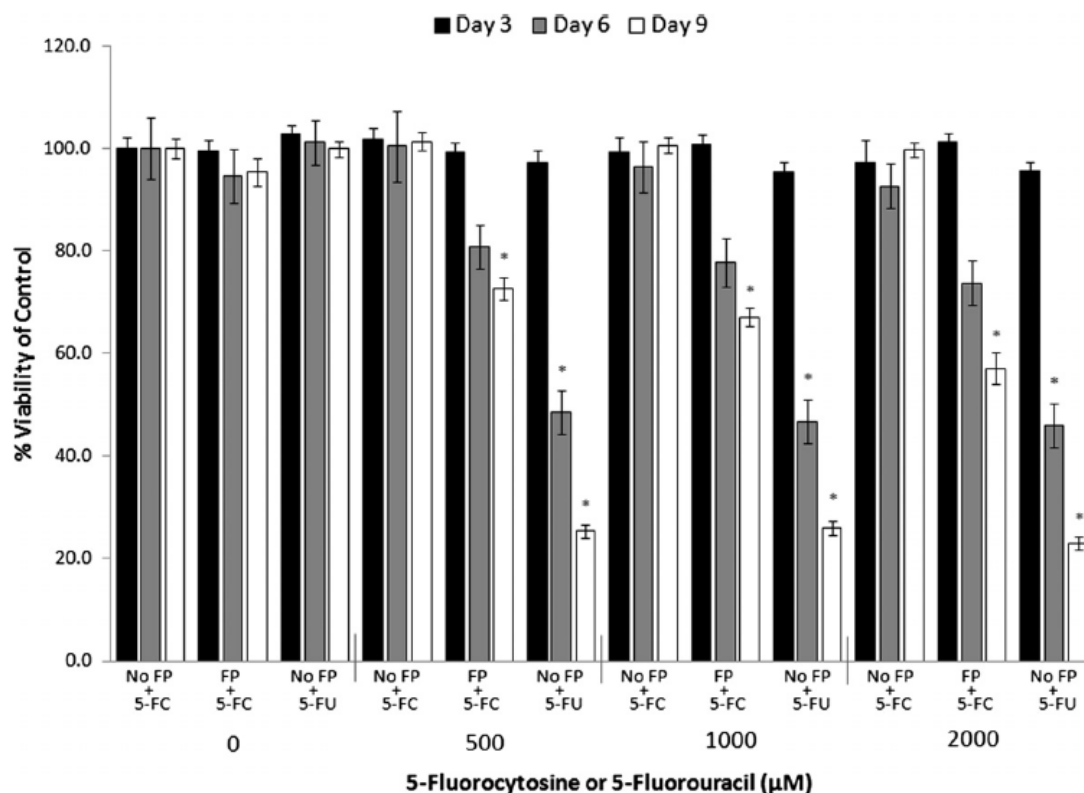


Fig. 5. Effect of 5-FC conversion to 5-FU on HAAE-1 endothelial cells. Cell viability was assessed using the Alamar Blue assay and normalized to the control (i.e. no FP and no 5-FC). A one-way ANOVA was performed for statistical analysis. Cells treated with the FP or cells treated with only 5-FU were compared to cells with no FP on the same day at the same 5-FC concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean \pm SE ($n = 3$).

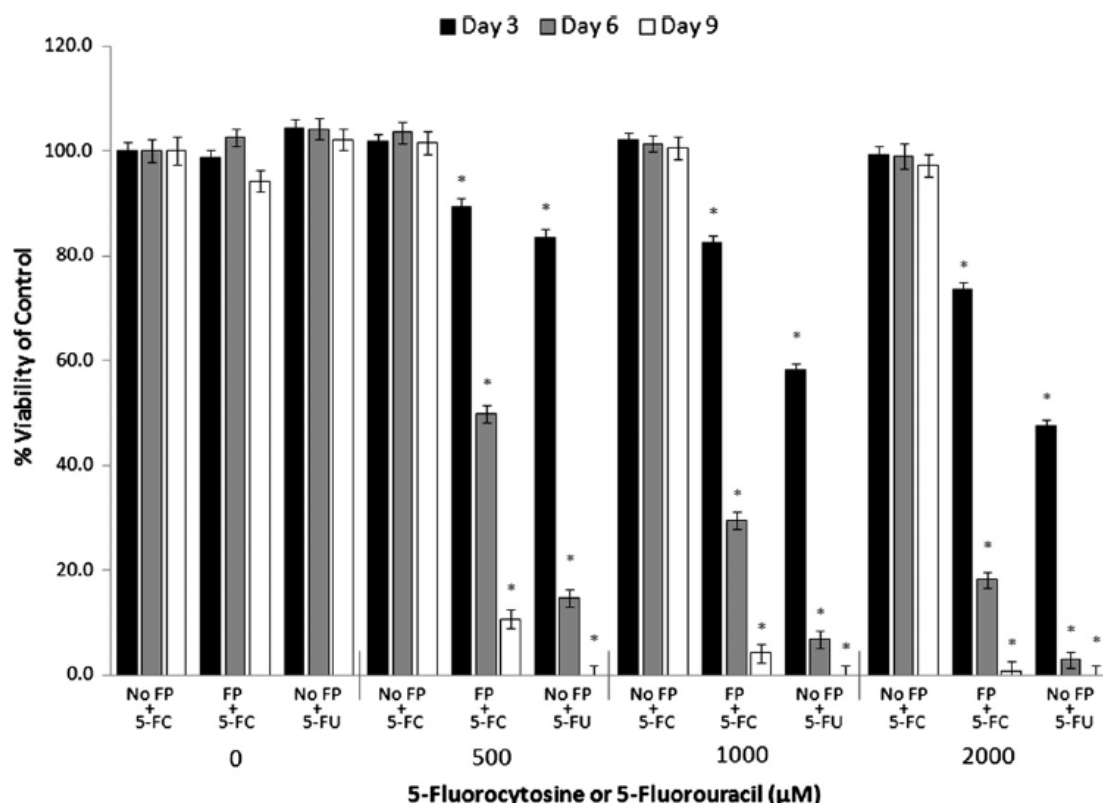


Fig. 6. Effect of 5-FC conversion to 5-FU on MCF-7 breast cancer cells. Cell viability was assessed using the Alamar Blue assay and normalized to the control (i.e. no FP and no 5-FC). A one-way ANOVA was performed for statistical analysis. Cells treated with the FP or cells treated with only 5-FU were compared to cells with no FP on the same day at the same 5-FC concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean \pm SE ($n = 3$).

treatment with commercially available 5-FU was performed. The viability data is presented as the percentage of fluorescence produced relative to control samples (no FP and 0 μ M 5-FC). All of the experimental groups were compared to the control for that particular day (no FP and the same 5-FC concentration). For all three cell lines, no significant cell death was found with 5-FC present and FP absent, or with 5-FC absent but FP present.

For the endothelial cells, there was negligible cell killing in the first 3 days of the experiments; however, significant killing ($p < 0.001$) was observed on day 9 for cells with FP plus 5-FC and on days 6 and 9 for cells with 5-FU. Killing was observed to be dose-dependent. At the same 5-FC concentration and incubation time, producing 5-FU using the FP and 5-FC gave less cytotoxicity than with 5-FU directly.

The two breast cancer cell lines exhibited similar patterns of cell death. Both MCF-7 and MDA-MB-231 cells receiving the FP plus 5-FC showed significant cell killing that was dose-dependent, compared to the control group, on days 3, 6, and 9 (Figs. 6 and 7, $p < 0.001$). By day 9, treatment with the FP plus 5-FC resulted in less than 10% viability at 500 μ M 5-FC for MCF-7 cells and at 1000 μ M 5-FC for MDA-MB-231 cells. As for the endothelial cells, the combination of 5-FC and the FP gave less cytotoxicity than with 5-FU directly at the same 5-FC concentration and incubation time.

4. Discussion

The major finding to emerge from this study is that CD-annexin V fusion protein in combination with the prodrug 5-FC has significant cytotoxic activity against endothelial cells and two breast cancer cell lines that expose PS on

their surface. This means that this system has the potential for a dual effect of cutting off the tumor's blood supply and acting directly on cancer cells as a result of the EPR effect that will allow the FP to be transported from the bloodstream to the tumor, while minimizing damage to normal organs and tissue.

The yield and purity of the FP are very good (50 mg per liter of culture broth and >96% purity, respectively), and its specific activity of 18 U/mg is consistent on a molar basis with that previously reported for purified yeast CD of 56 U/mg [24]. The theoretical molecular masses of the FP and CD are 106 kDa and 34, respectively. Therefore, the specific activity we would expect for the FP based on the previously reported specific activity of CD and the two molecular masses is 18 U/mg, or exactly what we measured.

Based on the size of the FP, the diameter of this molecule is approximately 7 nm, assuming a sphere with a density of 1 g cm⁻³ [25]. Blood vessel gaps and fenestrations in solid tumors are known to be leaky and range from 100 to 600 nm [26]. Based on this data, it is expected that in addition to the FP binding to surface of the vascular endothelial cells in the tumor, the FP will pass through the vascular wall and bind to the surface of tumor cells directly. When administered intravenously, 5-FC will be able to reach the FP, whether it is in the primary tumor or a distant

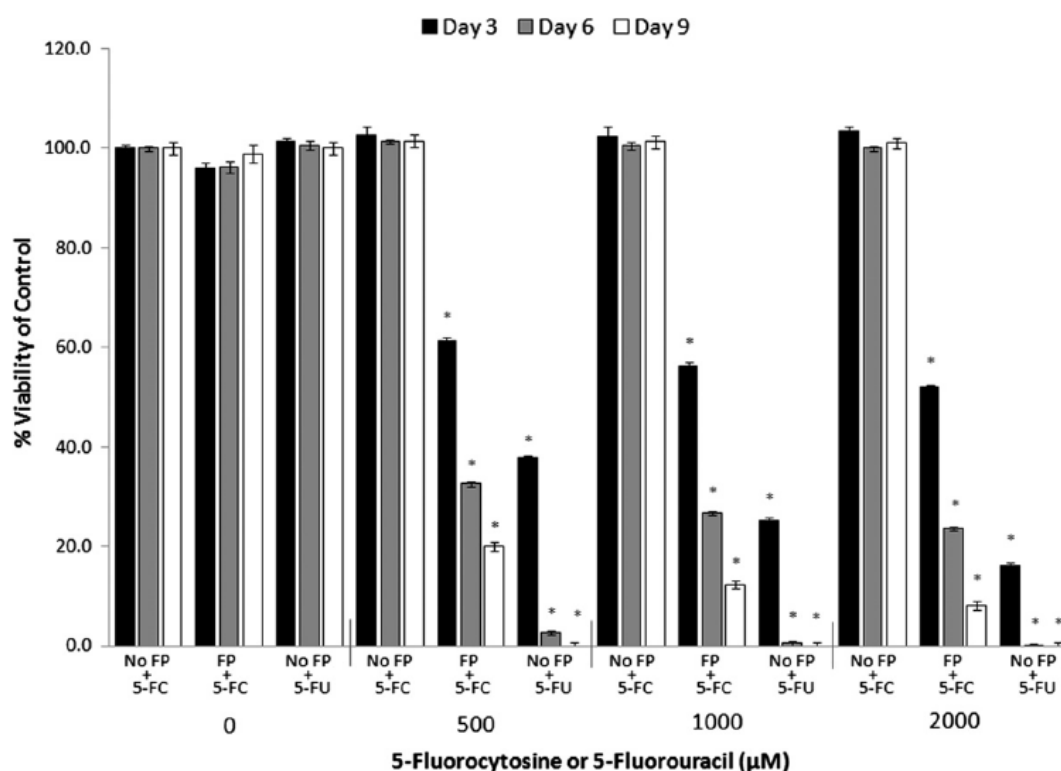


Fig. 7. Effect of 5-FC conversion to 5-FU on MDA-MB-231 breast cancer cells. Cell viability was assessed using the Alamar Blue assay and normalized to the control (i.e. no FP and no 5-FC). A one-way ANOVA was performed for statistical analysis. Cells treated with the FP or cells treated with only 5-FU were compared to cells with no FP on the same day at the same 5-FC concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean \pm SE ($n = 3$).

metastasis. Formation of 5-FU at the endothelial or cancer cell surface will result in uptake of 5-FU and the cascade of events ending in inhibition of RNA and DNA processes and cell death.

Critical for the success of this enzyme prodrug system, the binding strength of FP to externally positioned PS was determined to be in the low nanomolar range for endothelial cells, indicating high affinity for PS. Literature values of annexin V alone binding to endothelial cells have been reported from 2.7 to 15.5 nM [27,28]. The K_D values for the three cell lines studied were either below or the low end of this range of previously reported values, which may be the result of the FP existing as a dimer. The K_D values are also comparable to that of a different fusion protein we produced containing annexin V at the carboxy-terminus [23]. Additionally, we produced annexin V alone and found that it had a similar K_D [23].

Through the use of fluorescence microscopy, we observed that the FP was bound to the surface of breast cancer cells with PS exposed and not internalized (Fig. 3). By remaining on the cell surface and not being internalized, where it would be digested, the FP is able to convert 5-FC to 5-FU and thus lead to cytotoxicity of the cell and other cells surrounding it, by a bystander effect. Previous research has shown that annexin V by itself (i.e. not fused

to another protein) is taken up by endocytic vesicles after a two-dimensional crystallization on the cell surface [29]. Based on the images in Fig. 3, we can conclude that annexin V being part of a fusion protein has prevented it from forming two-dimensional crystals and then being internalized.

This study was able to mimic the extent of PS exposure previously found *in vivo* in both the normal vasculature and tumor vasculature [14,15]. When the endothelial cells were 100% confluent, no binding of the FP was observed in both the equilibrium binding assay and the microscopic fluorescence assay, indicating no PS exposure as is characteristic of normal vasculature; while for endothelial cells that were dividing and non-confluent, a strong binding of the FP was observed in the equilibrium binding assay, and binding was visualized at the cell surface using fluorescent FP.

When the endothelial cells were 100% confluent, no binding of the FP was observed in both the equilibrium binding assay and the microscopic fluorescence assay. Thus, our tests with 100% confluent endothelial cells mimic the normal vasculature, where it has been found that PS is not exposed on the endothelial cell surface *in vivo* [14,15].

Comparison between the efficacy of the FP/5-FC treatment that generates 5-FU and treatment with 5-FU by

itself at the same concentration showed that there was higher toxicity with 5-FU alone (Figs. 5–7). This finding is consistent with other reports comparing a drug's toxicity with the toxicity of the drug generated from its prodrug [13,30].

It is interesting to compare our results with another study in which two cancer cell lines were targeted using a fusion protein consisting of a single chain anti-CEA antibody linked to the amino-terminus of yeast CD [6]. Mel 5P human melanoma and LoVo human colon adenocarcinoma cells were incubated with this fusion protein, washed, and then incubated for 4 days with 5-FC at various concentrations. At the highest 5-FC concentration of 780 μ M, the survival of both cell lines was approximately 70%. By contrast, in our study for incubation for 3 or 6 days and 5-FC concentration of 500 or 1000 μ M, the survival for MCF-7 and MDA-MB-231 cells was in the range of 29–89% and 26–61%, respectively. From our study, it is evident that it is necessary to allow sufficient time for the enzyme prodrug therapy in order to achieve complete or near complete cell killing, which is likely because this therapy is dependent on targeting cells that are dividing. After 9 days of treatment with the CD-annexin V/5-FC enzyme prodrug therapy, the degree of cell death was very high, with 4% and 12% cell viability at 1000 μ M 5-FC for MCF-7 and MDA-MB-213 cancer cells, respectively, and with no significant toxicity without the FP at this same 5-FC concentration.

We have recently tested another novel enzyme prodrug system that consisted of L-methioninase-annexin V/selenomethionine, where the selenomethionine is converted to toxic methylselenol by the enzyme [23]. Similar cytotoxicity results were obtained using the same three cell lines. The presently discussed system is more of a known type of treatment, since 5-FU has been used clinically much more than methylselenol, while the L-methioninase-annexin V/selenomethionine system has the advantage that it also degrades L-methionine, which leads to death of methionine-dependent cancer cells.

Previous analyses have been done to determine the half-life of 5-FC and 5-FU. Patients given 5-FC orally with normal kidney function reportedly had a 5-FC half-life of 3–4 h, while those with poor renal function extended the half-life out to several days [4,31]. 5-FU has a very short biological half-life, found to be only 5–10 min [32]. Even with short life in plasma, studies have shown accumulation of 5-FU in tumor tissues, while no accumulation in normal tissues (not rapidly proliferating tissue) of rabbits was observed [33]. Since 5-FU is generated *within* the tumor using the cytosine deaminase-annexin V/5-FC system, side effects such as bone marrow suppression are expected to be low or nonexistent. Also, having a prodrug with a relatively long half-life will allow for the frequency of prodrug administration to be reduced, which can mean fewer hospital visits for patients undergoing treatment with this enzyme prodrug system.

Following administration of the FP, an immune response may occur, as is normal for any foreign molecule. PEGylation of CD has been done previously with little change to the enzyme properties [34]. An FP consisting of PEGylated CD linked to human annexin V would not be expected to produce an immune response; *in vivo* tests would be required to confirm this.

In summary, we have constructed a fusion protein consisting of a protein used to target exposed PS and CD enzyme that converts prodrug 5-FC to its toxic metabolite 5-FU. Recombinant production resulted in a highly active enzyme with functional annexin V, demonstrated by its ability to bind to PS exposed by endothelial cells and breast cancer cells. Cytotoxicity experiments verified this novel enzyme prodrug system has the ability to produce therapeutic levels of 5-FU and thus appears promising as a means of eliminating breast tumors wherever they occur in the body with minimal side effects.

Acknowledgements

This study was supported by a grant from the Department of Defense Breast Cancer Research Program (Idea Award W81XWH-08-1-0722). Brent Van Rite was partially supported by the U.S. Department of Education Graduate Assistance in Areas of National Need Program.

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APPENDIX V

Meeting Abstracts

- Brent D. Van Rite, Mohamad Cherry, Carla Kurkjian, Vassilios Sikavitsas, and Roger G. Harrison, “Targeted Enzyme Prodrug Therapy to Treat Breast Cancer,” Biomedical Engineering Society Annual Meeting, Hartford, CT, October, 2011.

Introduction

Cancer treatment is often dictated by the toxicity of the anticancer agent and the side effects it produces. A novel enzyme prodrug therapy has been created using a fusion protein containing annexin V to target only tumor vascular endothelial cells and cancer cells, reducing unwanted toxicity to healthy tissue. Methioninase enzyme will convert an inert prodrug selenomethionine to toxic methylselenol and also deplete the cancer cells of methionine needed for continued growth. *In vitro* studies have confirmed the ability of this enzyme prodrug system to kill cancer and endothelial cells, thus warranting testing in mice carrying breast tumor xenografts. The objective of this study it to test this new enzyme prodrug treatment for significantly reducing the growth of implanted breast cancer cells in nude mice.

Materials and Methods

Recombinant technology was used to express and purify methioninase-annexin V fusion protein. Pharmacokinetic analysis of the fusion protein was performed. A cytotoxic evaluation of the prodrug selenomethionine was done to determine a non-toxic dosage in mice. Following inoculation of MDA-MB-231 cancer cells, breast tumors were allowed to grow to a minimum size to assure formation of tumor vasculature. Tumor treatment with vehicle only, vehicle + selenomethionine, fusion protein only, and fusion protein + selenomethionine were completed and tumor response was documented by monitoring tumor size. MDA-MB-231 cells expressing green fluorescent protein were used to monitor metastasis of cells to the lungs and liver.

Results and Discussion

Recombinant methods have produced an active fusion protein of high purity for use in nude mice. Pharmacokinetic testing revealed complete clearance from the bloodstream to occur within 8 hours of intraperitoneal injection. Selenomethionine levels up to 12 mg/kg were shown to cause no apparent toxicity, while higher levels were lethal. Preliminary testing of the enzyme prodrug system demonstrated a reduction in tumor growth rate of 80% compared to the vehicle only control group.

Conclusions

A methioninase-annexin V fusion protein was purified from *E. coli* and demonstrated complete clearance by 8 hours. Selenomethionine toxicity did not occur until above 12 mg/kg. The results obtained to date of treating nude mice with this enzyme prodrug treatment are promising. Additional tests in mice of this treatment that incorporate a methionine-deficient diet are warranted.

Acknowledgments

The authors acknowledge the support of the Department of Defense Breast Cancer Research Program and the Department of Education Graduate Assistance in Areas of National Need Program.

- Van Rite, B.D., Lazrak, Y.A., Pagnon, M., McFetridge, P.S., Sikavitsas, V.I., Bose, P., Kurkjian, C., and Harrison, R.G. (speaker), “Novel Enzyme Prodrug Therapy of Breast Cancer Designed for Effective Delivery to the Tumor,” Era of Hope 2011 Breast Cancer Research Conference, Orlando, FL, August, 2011.

A new anticancer therapy for solid tumors is being developed to eliminate both the primary tumor and distant metastases. This approach uses an enzyme prodrug system targeted to the solid tumor vasculature, specifically to exposed phosphatidylserine. Two recombinant exogenous enzymes (L-methioninase and cytosine deaminase) were linked separately to human annexin V protein to form fusion proteins (FPs). Annexin V will specifically target both the outer membrane of endothelial cells in tumor vasculature and cancer cells. The resulting therapeutic effects are (1) death to tumor endothelial cells leading to vessel clotting and oxygen deprivation, and (2) death to surrounding tumor cells due to leaky capillaries and the EPR effect. Cytotoxicity experiments have verified the ability of the FPs to produce therapeutic levels of methylselenol and 5-fluorouracil *in vitro*. Preliminary tests in nude mice using targeted L-methioninase and selenomethionine prodrug gave promising results.

- Van Rite, B.D., Lazrak, Y.A., Pagnon, M., McFetridge, P.S., Sikavitsas, V.I., Bose, P., Kurkjian, C., and Harrison, R.G. (speaker), “Novel Enzyme/Prodrug for Targeting and Treatment of Solid Breast Tumors,” American Institute of Chemical Engineers Annual Meeting, Salt Lake City, November, 2010.

A novel therapy for solid tumors is under development to treat primary tumors and distant metastases via an enzyme/prodrug system targeted specifically to solid tumor vasculature. The L-methioninase enzyme is linked to human annexin V protein in a fusion protein (FP). This therapy takes advantage of the fact that phosphatidylserine (PS), an anionic phospholipid, is exposed almost exclusively on the outer membrane of cancer and endothelial cells of tumor vasculature and that annexin V binds specifically to PS. Intravenous administration allows binding of the FP to the tumor vasculature, followed by delivery of the prodrug selenomethionine and conversion to cytotoxic methylselenol by L-methioninase. It is expected that this enzyme prodrug therapy will lead to the elimination of tumors because of a cutoff of the tumor's blood supply, as well by the cytotoxic effect of methylselenol on cancer cells and by methionine depletion of methionine-dependent cancer cells.

In vitro characterization of the FP showed a dissociation constant (K_d) of approximately 2 nM for human endothelial cells and MCF-7 and MDA-MB-231 breast cancer cells, indicating strong specific binding of annexin V to PS. *In vitro* cytotoxicity tests of enzyme prodrug treatment showed significant cell killing of the endothelial cells and the two breast cancer cell lines over a period of 3 days. Preliminary *in vivo* data will also be presented regarding FP pharmacokinetics and enzyme prodrug therapy of nude mice carrying breast tumor xenografts.

- Brent D. Van Rite, Yahya A. Lazrak, Magali Pagnon, Vassilios I. Sikavitsas, Prithvi Bose, Carla Kurkjian, and Roger G. Harrison, “A Novel Enzyme/Prodrug for Targeting and Treatment of Solid Breast Tumors,” Biomedical Engineering Society Annual Meeting, Austin, TX, October, 2010.

A novel therapy for solid tumors is under development to treat primary tumors and distant metastases via an enzyme/prodrug system targeted specifically to solid tumor vasculature. The L-methioninase enzyme is linked to human annexin V protein in a fusion protein (FP). This therapy takes advantage of the fact that phosphatidylserine (PS), an anionic phospholipid, is exposed almost exclusively on the outer membrane of cancer and endothelial cells of tumor vasculature and that annexin V binds specifically to PS. Intravenous administration allows binding of the FP to the tumor vasculature, followed by delivery of the prodrug selenomethionine and conversion to cytotoxic methylselenol by L-methioninase. Binding of the FP leads to the following effects: (1) death of tumor endothelial cells from the methylselenol generated, clotting the tumor vasculature and cutting off oxygen to the tumor, (2) death of tumor cells by the action of methylselenol, (3) death of methionine-dependent tumor cells by methionine depletion.

In vitro characterization of the FP showed a dissociation constant (K_d) of 2 nM for human endothelial cells and breast cancer cells MCF-7 and MDA-MB-231, indicating strong specific binding of annexin V to PS. Binding stability tests determined the FP stays bound on the endothelial cells for at least 3 days and cytotoxicity tests using the Alamar Blue assay revealed significant cell killing over a period of 3 days. Preliminary *in vivo* data will be presented regarding FP pharmacokinetics and treatment of nude mice carrying breast tumor xenografts.

- Brent D. Van Rite (speaker), Magali Pagnon, Yahya Lazrak, and Roger G. Harrison, “The Use of Targeted Enzyme/Prodrugs for the Treatment of Solid Tumors,” 39th Annual Biochemical Engineering Symposium, Kansas State University, Manhattan, KS, April, 2010.

A new anticancer therapy for solid tumors is being developed to eliminate both the primary tumor and distant metastases. This approach uses an enzyme/prodrug system targeted to the solid tumor vasculature. Two different genes encoding bacterial enzymes were linked to human annexin V protein to form fusion proteins (FPs). Annexin V is known to bind with high affinity and specificity to exposed the anionic phospholipid phosphatidylserine (PS) on the outer membrane of endothelial cells in the tumor vasculature. The enzymes are used to activate nontoxic prodrugs into toxic anticancer agents. Following FP administration into the bloodstream, the prodrugs are delivered, and the resulting toxic molecules induce (1) death to tumor endothelial cells leading to vessel clotting and oxygen deprivation, and (2) death to surrounding tumor cells because of leaky capillaries and the enhanced permeability and retention (EPR) effect. Using recombinant bacterial technology, the fusion proteins were expressed and purified from *E. coli*. Assays were performed to determine the strength of FP binding, cytotoxicity and binding stability for human endothelial cells and breast tumor cell lines MDA-MB-231 and MCF-7. Cytotoxicity assays demonstrating cell killing were done using the Alamar Blue assay. Near complete killing was observed for both the the methioninase/selenomethionine and the cytosine deaminase/5-fluorocytosine enzyme prodrug combinations.

- Yahya A. Lazrak, Brent D. Van Rite, Magali Pagnon, Arafat Tfayli, Peter S. McFetridge, and Roger G. Harrison (speaker), “Targeting the Vasculature of Solid Tumors with Enzyme/Prodrug and Methionine-Depletion Therapy,” American Institute of Chemical Engineers Annual Meeting, Nashville, November, 2009.

A new anticancer therapy for solid tumors is being developed to eliminate both the primary tumor and distant metastases. This approach uses both enzyme/prodrug and methionine-depletion therapy. The L-methioninase enzyme is linked to the human annexin V protein in a fusion protein (FP). This therapy takes advantage of the fact that phosphatidylserine (PS), an anionic phospholipid, is exposed almost exclusively on the outer membrane of cancer and tumor vasculature endothelial cells and that annexin V binds specifically to PS. After the FP administered to the bloodstream binds to the tumor vasculature, the prodrug selenomethionine is administered and converted by L-methioninase to a cytotoxic compound, methylselenol. Binding of the FP will lead to the following effects: (1) death of tumor endothelial cells from the methylselenol generated, causing clotting of the tumor vasculature and a cutoff of oxygen to the tumor, (2) death of tumor cells by the action of methylselenol, (3) death of methionine-dependent tumor cells by the depletion of methionine.

Using cells grown in vitro, binding assays were performed with the FP to determine the strength and specificity of binding, and cytotoxicity assays were carried out using the enzyme/prodrug treatment. The dissociation constant of the FP was found for both human endothelial cells and breast cancer cells, and the dissociation constant values indicated relatively strong binding. An in vitro binding stability test determined that the FP remained bound on the endothelial cells for at least 3 days. Cytotoxicity data for both endothelial and breast cancer cells was obtained for the effect of adding selenomethionine prodrug to cells for which the FP is bound on the cell surface. Cell viability was evaluated using the Alamar blue assay and cell counting.

- Harrison, R.G., Van Rite, B.D., Lazrak, Y.A., Pagnon, M., and McFetridge, P.S., “Targeting of Solid Tumor Vasculature with Enzyme/Prodrug and Methionine-Depletion Therapy,” Biomedical Engineering Society Annual Meeting, Pittsburgh, October, 2009.

A new anticancer therapy for solid tumors is being developed to eliminate both the primary tumor and distant metastases. This approach uses both enzyme/prodrug and methionine-depletion therapy. The L-methioninase enzyme is linked to the human annexin V protein in a fusion protein (FP). This therapy takes advantage of the fact that phosphatidylserine (PS), an anionic phospholipid, is exposed almost exclusively on the outer membrane of cancer and tumor vasculature endothelial cells and that annexin V binds specifically to PS. After the FP administered to the bloodstream binds to the tumor vasculature, the prodrug selenomethionine is administered and converted by L-methioninase to a cytotoxic compound, methylselenol. Binding of the FP will lead to the following effects: (1) death of tumor endothelial cells from the methylselenol generated, causing clotting of the tumor vasculature and a cutoff of oxygen to the tumor, (2) death of tumor cells by the action of methylselenol, (3) death of methionine-dependent tumor cells by the depletion of methionine.

Binding assays were performed with the FP to determine the strength and specificity of binding. The specific binding dissociation constant (K_d) of the FP was found to be 2 nM for both

human endothelial cells and MCF-7 breast cancer cells grown *in vitro*, indicating strong specific binding of annexin V to PS. An *in vitro* binding stability test determined the FP stays bound on the endothelial cells for at least 3 days. *In vitro* cytotoxicity tests have been done using the Alamar Blue assay to demonstrate cell killing.

- Lazrak, Y.A. (speaker), Neves, L.F.F., McFetridge, P.S., and Harrison, R.G., “Novel Enzyme-Prodrug Therapy for Cancer,” oral presentation, Biomedical Engineering Society Annual Meeting, St. Louis, October, 2008.

We are developing a new type of enzyme-prodrug therapy designed to selectively clot the vasculature of tumors and kill cancer cells. It has been shown that the anionic phospholipid phosphatidylserine (PS) is exposed almost exclusively on the external membrane layer of cancer cells and endothelial cells of the tumor vasculature. It is also known that the human protein annexin V binds specifically and strongly to PS. We have engineered a fusion protein (FP) containing annexin V linked to the enzyme methioninase, which has the ability to convert a biologically safe compound, selenomethionine, to a toxic compound, methylselenol. The annexin V part of the FP will specifically bind to the tumor vasculature, while the methioninase part will convert the selenomethionine prodrug to the toxic methylselenol leading to three mechanisms to kill the tumor: 1) death of the endothelial cells causing cloture of tumor vasculature, thus cutting off the oxygen supply of the cancer cells; and 2) methylselenol will be transported to tumor cells by diffusion and fluid permeation resulting in their death.

We have studied the binding of purified FP to PS immobilized on microtiter plates and to human endothelial cells in which PS has been induced to the on the cell surface by the addition of a low level of hydrogen peroxide. Data has also been obtained for the effect of adding selenomethionine to endothelial cells *in vitro* in which the FP is bound on the cell surface. The cell viability is evaluated by performing the Alamar Blue assay, cell counting, and cell observation with a microscope.

- Harrison, R.G. (speaker), Lazrak, Y.A., Neves, L.F.F., McFetridge, P.S., and Tfayli, A., “Novel Enzyme-Prodrug Therapy for Cancer,” American Institute of Chemical Engineers Annual Meeting, Philadelphia, November, 2008.

We are developing a new type of enzyme-prodrug therapy designed to selectively clot the vasculature of tumors and kill cancer cells. It has been shown that the anionic phospholipid phosphatidylserine (PS) is exposed almost exclusively on the external membrane layer of cancer cells and endothelial cells of the tumor vasculature. It is also known that the human protein annexin V binds specifically and strongly to PS. We have engineered a fusion protein (FP) containing annexin V linked to the enzyme methioninase, which has the ability to convert a biologically safe compound, selenomethionine, to a toxic compound, methylselenol. The annexin V part of the FP will specifically bind to the tumor vasculature, while the methioninase part will convert the selenomethionine prodrug to the toxic methylselenol leading to two mechanisms to kill the tumor: 1) death of the endothelial cells causing cloture of tumor vasculature, thus cutting off the oxygen supply of the cancer cells; and 2) methylselenol will be transported to tumor cells by diffusion and fluid permeation resulting in their death.

We have studied the binding of purified FP to PS immobilized on microtiter plates and to human endothelial cells in which PS has been induced to the on the cell surface by the addition of a low level of hydrogen peroxide. Data has also been obtained for the effect of adding selenomethionine to endothelial cells *in vitro* in which the FP is bound on the cell surface. The cell viability is evaluated by performing the Alamar Blue assay, cell counting, and cell observation with a microscope.